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Comparative virulence of *Salmonella cholerae-suis* var *kunzendorf* strains

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PREFACE

This dissertation is divided into five separate but related papers. Each paper is presented separately with individual summaries, materials and methods, references, etc. A review of the literature concerning the mechanisms of bacterial virulence precedes these and a supplemental reference list pertaining to this review appears on pages 18-27.

SUMMARY

Seventy-two field strains of smooth Salmonella cholerae-suis var kunzendorf were examined for susceptibility to killing by antibody-complement (Ab-C) after they were exposed to Tris-EDTA, lysozyme, or saline solution. Considerable differences were found among the strains with some being susceptible to killing under all conditions and some being completely resistant. Evidence was obtained to indicate that Tris-EDTA may inhibit the growth of S. cholerae-suis in addition to its documented effect on the release of lipopolysaccharide from the cell wall of gram-negative bacteria. Twenty-two of these strains were further evaluated for their susceptibility to killing by porcine peripheral blood polymorphonuclear neutrophils (PMNs) under various conditions. Susceptibility to PMN killing was found to correlate well with the Ab-C susceptibility of these strains. The mouse virulence of these strains was examined. Eight of the strains were selected for further evaluation in the pig. With one exception, the virulence of these strains for pigs was well predicted by the results of the in vitro Ab-C bacteriolysis. Mouse virulence was also found to correlate with pig virulence but was somewhat less accurate. The immune response of the pig to one of the strains of S. cholerae-suis was evaluated with and without the presence of the immunosuppressive drug cyclophosphamide. In addition the effects of CY on the delayed hypersensitivity (DH) reaction to Mycobacterium avium were examined. For this, pigs were sensitized with M. avium sensitinogen. Pigs given 20 mg/kg of CY on days 0, 2 and 4 and

infected with 10^6 S. cholerae-suis on day 0 were more severely affected than those given the same dose of CY and infected on day 4. The DH response in the former group was equivalent to the non-sensitized group and thus was severely depressed. The DH reaction of pigs infected on day 4 was also decreased compared to the sensitized controls. Mortality rates and serological responses were compared for the various groups of pigs. The virulence and immunogenicity of several mutants of S. cholerae-suis were evaluated in mice. An aro$^-$ galE mutant of strain 38 proved to be reduced in virulence from the parent strain but provided only moderate protection against homologous challenge. Thymidine-requiring mutants of strains 9, 33, 38, 51 and 61 were observed to be avirulent for mice. All provided as much or more protection against challenge than the parent strain.

LITERATURE REVIEW

An effective immune response against a bacterial pathogen is dependent on many factors. Attempts at elucidation of these factors have commanded a great deal of time and effort on the part of animal health workers especially during the past 100 years. Control of those bacterial pathogens for which control measures have been developed has evolved around relatively simple techniques first used by Pasteur with fowl cholera and anthrax. Most immunizing agents against bacterial diseases rely upon either living avirulent vaccines, killed bacteria (bacterins), or altered bacterial toxins (toxoids). Bacterins and toxoids have been useful in some diseases.[1] Organisms which possess the means for intracellular survival and multiplication have been best controlled by living avirulent strains of bacteria. However, there exists a fine distinction between virulence and immunogenicity for most isolates of bacteria. Those which are totally avirulent are frequently poorly immunogenic. Those which are highly immunogenic are often fully virulent. Rough strains of bacteria have been employed as immunizing agents with some success but smooth strains of bacteria appear to provide a much higher level of immunity.[2] It has been demonstrated that smooth strains of bacteria possess antigens which are qualitatively and/or quantitatively different from corresponding rough strains.[3,4] Such differences markedly alter the sensitivity of organisms to killing by antibody and complement. In addition, the phagocytosis and killing by polymorphonuclear leukocytes and macrophages is much more effective in bacteria demonstrating rough

characteristics. This altered sensitivity also affects the virulence of these organisms for experimental animals. Even among smooth organisms of the same species there exist qualitative and quantitative differences which alter the virulence of these organisms. A means of detecting these differences would assist in selection of potential avirulent immunizing strains of living bacteria.

Virulence as a property of bacteria involves highly diverse mechanisms and debate exists as to the exact nature of some of these mechanisms. Exotoxins have been classically a virulence mechanism of gram-positive bacteria. Potent exotoxins are described for Clostridia, Corynebacterium diphtheriae and Bacillus anthracis.[5] Endotoxins have classically been described as a virulence mechanism of gram-negative organisms such as Escherichia coli, Salmonella sp., and Hemophilus sp. The classical divisions break down when one considers the exotoxins such as the enterotoxins of Vibrio cholerae and E. coli and the neurotoxin of Shigella dysenteriae.[6]

Closely related to mechanisms of virulence is the ability of a given bacterium to evade the immune response of the host. Facultative intracellular organisms such as Mycobacteria are seemingly unaffected by antibody. Other facultative intracellular organisms are affected by antibody but only in the extracellular phase. Extracellular bacteria such as Staphylococcus aureus, Streptococcus pyogenes and Pseudomonas aeruginosa are much more readily eliminated via antibody mediated bacteriolysis.

The majority of fully virulent, smooth strains of gram-negative

bacteria are highly refractory to the effects of Ab and complement *in vitro*. Reynolds and Pruul[7] described the use of the chelating agent EDTA to decrease the availability of divalent cations which are required for structural integrity of smooth gram-negative rods. Bacteria grown in the presence of EDTA were rendered much more susceptible to killing by Ab-C. The use of organic cations such as tris (hydroxymethyl) amino-methane (Tris) causes an increase in the cell wall permeability of smooth gram-negative bacteria which also results in the increased sensitivity to antibody and complement.[8] Lysozyme is a bacteriolytic enzyme present in mammalian sera and thought to play a role in the defense against bacterial agents.[9] Alteration of the cell walls of Ab-C resistant smooth gram-negative bacteria with EDTA, Tris, or lysozyme would permit a more in-depth study of the factors associated with resistance to Ab-C.

The ability of bacteria to resist the activity of phagocytic cells is also an important determinant of their virulence.[4] Lipopolysaccharide associated with smooth gram-negative organisms is believed to contribute greatly to this resistance. Capsular material when present in large quantities is thought to inhibit the phagocytosis and killing of certain bacteria.[10] Such capsular material has been demonstrated to inhibit the myeloperoxidase-hydrogen peroxide system in neutrophils and thereby inhibit killing of the ingested bacterium.

Another factor bearing on the virulence of bacteria is the efficiency of iron binding of a given bacterium. All microorganisms

with the possible exception of the lactic acid bacteria require iron.[11] In aerobic environments the availability of iron is restricted due to the tendency of iron to form large insoluble aggregates at neutral or alkaline pH.[12] To acquire the necessary iron, aerobic bacteria have evolved specialized iron-solubilizing and iron-chelating compounds known as siderophores.[13,14] Salmonella species[15] and other Enterobacteriaceae[16] secrete a phenolate-type siderophore termed enterochelin or enterobactin. A pathogen does not have to contend with insoluble ferric iron *in vivo* but it must obtain its iron from the host. Iron-binding proteins (transferrin and ferritin in serum, lactoferrin in secretions) sequester essentially all the iron in these environments.[17] The most likely mechanism employed by a pathogen for competing with these iron-binding proteins is via the siderophore. The presence of this compound has been demonstrated to be a virulence factor for *S. typhimurium*.[11]

Another mechanism of iron uptake is specified by the Col V plasmid. It involves iron chelation by an inducible hydroxymate siderophore.[18] Williams[19] demonstrated that iron uptake in *E. coli* specified by the Col V plasmid was an important virulence factor. A significant proportion of strains of *E. coli* from cases of septicemia in humans and domestic animals harbor Col V plasmids.[20] There are several Col V plasmid-associated characteristics which may be implicated in pathogenicity of *E. coli*. Bacteria carrying the Col V plasmid show enhanced ability to adhere to intestinal epithelium.[21] Colicin V itself may act synergistically with endotoxin to increase

vascular permeability[22] and depress macrophage activity.[23]

The presence of the Col V plasmid is not absolutely correlated with serum resistance in all cases. The gene specifying serum resistance, the iss determinant, was found to be closely linked to the Col V gene.[24] The iss gene product is thought to inhibit the insertion of the membrane attack complex of complement.[9] It appears that resistance to complement-mediated killing does not result from a block of the activation of the complement cascade. Indeed, much larger quantities of the terminal components C_5, C_7 and C_9 were bound to a smooth Salmonella minnesota strain S218 than were bound to Re595, a rough mutant of S218.[25,26] The binding of the terminal components reached a peak at 10 min and a progressive elution was noted thereafter. Comparatively few molecules of radiolabeled membrane attack complexes (MAC) were bound to the Re595 cells. The MACs could be eluted from the surfaces of the smooth S218 but not the rough Re595 after incubation in buffers of increasing ionic strength.[26] Thus, it appears that the MACs are not effectively inserted into the bacterial membranes and are released without causing lethal damage.[9] In contrast to serum susceptible organisms, phospholipid is not released from serum-resistant organisms. Thus, one mechanism of resistance to Ab-C is the failure of amphiphilic MACs to integrate into hydrophobic domains in the bacterial cell wall.[9]

Another factor related to the ability of MACs to integrate into membranes is the degree of fluidity of the membrane. Factors increasing the fluidity of either the outer or cytoplasmic membranes

of gram-negative bacteria may enable a more efficient assembly and integration of MACs.[9] Membrane fluidity may be obligatory for formation of functional complement lesions.[27] Removal of half of the S-type lipopolysaccharide from membranes by EDTA greatly increased the fluidity of these membranes. EDTA treatment has been shown to increase the susceptibility of gram-negative bacteria to complement-mediated killing.[7] Curiously, membranes become initially more rigid following insertion of MACs[28,29] probably as a result of reorientation of the lipid bilayer due to strong binding of phospholipid to the MACs.[30] It is possible that lipopolysaccharides and membrane proteins may mediate resistance to complement-mediated bacteriolysis by virtue of their effects on membrane fluidity.

In addition to its role in membrane fluidity, lipopolysaccharide is thought to affect the sensitivity of gram-negative bacteria to complement-mediated killing in other ways. These are a matter of some debate. Mutations from the smooth to the rough form, usually but not invariably associated with the loss of lipopolysaccharide O side chains[31-33] are accompanied by drastic increases in serum susceptibility.[34-38] Dlabac[39] demonstrated a progressive increase in serum susceptibility with increased loss of sugar residues from the lipopolysaccharide in a series of mutants derived from a smooth S. typhimurium strain. UDP-galactose-4-epimerase (galE) deficient mutants when grown in the absence of galactose form incomplete lipopolysaccharides which lack both O side chains and the portion of the core distal to the biosynthetic lesion.[40] When galactose is

supplied to such mutants they become increasingly resistant to serum with time. Resistance to serum increased in direct proportion to the amount of galactose added to the medium.[41]

Others have found that mutations to serum resistance occurred independently of quantitative changes in lipopolysaccharide content of E. coli.[38] No significant differences could be found in the amount of lipopolysaccharide extractable from 28 smooth urinary tract E. coli strains of differing serum susceptibility.[42] Many strains carrying a full complement of O side chains exhibit a delayed sensitivity to complement-mediated killing compared to rough strains.[43] The degree of serum resistance may be a reflection of variation in O side chain length and the degree of substitution of core stubs by O side chains. These O side chains per se may not determine resistance to complement-mediated killing but a high degree of substitution of lipopolysaccharide core stubs by long O side chains may result in delayed serum killing of smooth isolates.[9] The long O side chains may cause a physical separation of activated complement components from the site of lesion formation on the bacterial membrane. Intermediates may therefore decay before they can become incorporated into functional MACs.[44-47]

The absence of antibody against bacterial membrane components may not be a major determinant of the bactericidal effects of serum. Antibody-independent activation of complement by gram-negative bacteria has been demonstrated to take place via both alternate and classical complement pathway components. Purified bacterial

lipopolysaccharides activate both pathways independent of antibody.[48,49] and the lipid A portion has been demonstrated to bind and activate C1.[48,50] Antibody independent binding and activation of C1 has been shown to result in the formation of a functional MAC and killing of gram-negative bacteria.[51]

Acidic exopolysaccharides may play a role in the virulence of some organisms but this is apparently not well correlated with resistance to complement-mediated killing.[9] Polysaccharide capsules apparently do not represent a diffusion, permeability, or adsorption barrier to macromolecules such as immunoglobulins[52] or other proteins.[53] Interference with O agglutinability observed in organisms with thick capsules is thought to be due to surface protein components[54] or inhibition of lattice formation between adjacent bacteria.[55] Acidic exopolysaccharides probably play a role in masking antigenic components and may inhibit phagocytosis and killing of organisms by polymorphs or macrophages.

The resistance of gram-negative bacteria to phagocytosis and killing by polymorphonuclear neutrophils (PMNs) and macrophages appears to be less well understood than factors associated with resistance to complement-mediated killing. These two facets of the immune response appear to be interrelated and certain virulence factors appear to affect both. Resistance to ingestion is mediated primarily by surface components and appears to be the major mechanism utilized by some bacteria to avoid killing by phagocytes.[56] Resistance of E. coli to phagocytosis and killing by mouse

polymorphonuclear leukocytes (PMNs) apparently depends on the presence of a complete polysaccharide side chain in the cell wall O antigen. Genotypically or phenotypically induced defects of O antigen structure render E. coli much more susceptible to phagocytosis.[57] In Salmonella a complete polysaccharide core of the cell wall has been shown to be important for resisting ingestion and intracellular killing.[58] The presence of O specific side chains and the composition of these contributes further resistance.[3,4,57] The glycolipoprotein associated with the slime layer of Pseudomonas aeruginosa apparently exerts a major effect on PMNs.[59] The production of the glycolipoprotein and its diffusion from the site of infection are associated with a sharp drop in circulating PMNs [60] and sequestration of a PMN-glycolipoprotein complex in the liver.[61] The glycolipoprotein is also antiphagocytic in vitro.[62] It is apparent that many substances may be responsible for inhibition of phagocytosis and killing and that they may vary greatly between bacterial species.

It is clear that ingestion is closely tied to the opsonization of the bacterial agent.[63] Some ingestion occurs in the absence of specific opsonins but the efficiency of ingestion increases markedly with the degree of opsonization. Adherence is the first step in the process of ingestion. Nonspecific interactions may occur such as with polystyrene beads or very specific interaction may occur, such as the interaction of a ligand with a particular cell surface binding site.[64] The examples of the latter most often studied are those of antibodies or complement. Receptors for the Fc portion of immunoglobulin

molecules have been recognized on all mononuclear phagocytes examined so far.[65-70] In addition, most phagocytic cells exhibit receptors for C_3b.[71] In macrophages immunoglobulin receptors consistently mediate ingestion while C_3 receptors only mediate ingestion by certain "activated" cells.[72] In many cases, the mechanism of bacterial adherence to the phagocyte has not been well studied or has not been examined from the perspective of specific binding structures.[56] The particular mechanism by which binding occurs may be very significant in determining the ultimate fate of the ingested organism. The internalization of intracellular parasites may proceed by a mechanism totally different from that observed in phagocytosis.[73] However, many intracellular parasites enter cells via phagocytic mechanisms and are able to survive intracellularly because of other factors.[56]

An important determinant of whether a particular bacterium is phagocytosed is the contact angle (θ) of the bacterium compared with that of the θ of the phagocytic cell.[74] Contact angles greater than 18° allow ready phagocytosis; those less than 18° inhibit phagocytosis. The actual determinant of the ease of phagocytosis is whether a bacterium is more hydrophobic or hydrophilic than the phagocytic cells. Those more hydrophobic are readily phagocytosed and those more hydrophilic are able to resist phagocytosis to a greater degree. Smooth bacteria having a full complement of O side chains tend to be more hydrophilic.

Certain bacteria are able to avoid, inactivate or resist the bactericidal mechanisms present inside the phagocyte. The classical

example is that of Mycobacterium tuberculosis in which the living organism is able to impair the fusion of the phagosome with the lysosome.[75] The presence of antibody on the surface of the organism abrogates the impairment of phagosome-lysosome fusion.[76] However, viability and subsequent multiplication of M. tuberculosis exposed to lysosomal enzymes was not affected, indicating yet another mechanism of resistance to killing.

Bacteria have been observed to inhibit the generation of reduced oxygen derivatives such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), or hydrogen peroxide (H_2O_2). The generation of these compounds via the granule-associated enzyme myeloperoxidase is thought to be a very important bactericidal mechanism of the PMN when formed in the presence of an anionic co-factor such as iodide or chloride.[77,78] Salmonella typhi has been shown to impair oxygen radical killing mechanisms in PMNs.[79] PMNs ingesting virulent S. typhi exhibited a significantly smaller increase in postphagocytic oxidative metabolism than PMNs ingesting avirulent S. typhi.[80] It was postulated that the virulent S. typhi failed to stimulate receptors that trigger the PMN oxidative metabolism. This may also apply to mononuclear phagocytes which play an important role in the ultimate fate of a host infected with Salmonella.

One of the most important determinants of the ability of a bacterial agent to produce disease is its ability to adhere to the site of infection. Many organisms such as streptococci and staphylococci exhibit a distinct tissue tropism and can be found

adhered to specific areas of the host's body.[81] A fibrillar network of lipotechoic acid-protein complexes appears to constitute the surface adhesin of Streptococcus pyogenes. Presumably adhesins vary from organism to organism as indicated by the differing tropisms.

One of the most important mechanisms of bacterial adherence involves the production of pilus antigens by gram-negative bacteria. Most pili are composed of protein subunits.[82] A specific type of pilus (Type 1) is common among the Enterobacteriaceae.[82,83] Most Salmonella isolates from natural sources possess type 1 pili.[84,85] Some pili bind to a mannose receptor on various cell surfaces and are susceptible to treatment with α-mannosidase.[86] These are termed mannose sensitive receptors.[87] In addition there exist receptors for many other molecules such as galactose and α-N-acetylgalactosamine.[88] These are termed mannose resistant. The nature of many of the mannose resistant receptors remains unknown[89,90] They are probably quite diverse. The mannose resistant receptors are important mediators of adhesion in urinary tract infections and intestinal infections with enteropathogenic E. coli.[89,91] As such they are important factors in the establishment of an infection in these tissues.[92]

Enteropathogenicity of E. coli isolates in neonatal diarrhea in pigs is highly correlated with the presence of plasmid-mediated K88 and K99 pilus antigens.[93-95] K99 and a third pilus antigen (987P) mediate colonization of the ileum in the pig whereas K88 mediates colonization of the entire small intestine. Pilus antigens are very frequent among isolates of E. coli from pyelonephritis in humans.[96]

The adherence of Neisseria gonorrheae to various areas of the human body is a pilus-mediated phenomenon.[97] Thus, even though pilus antigens may not be important in mechanisms of virulence per se, they are apparently very important determinants of the establishment of infection. As such, the production of immunizing agents containing pilus antigens have met with some success.[94]

One of the most important antiphagocytic mechanisms associated with virulence is the production of capsules and slime layers.[98] Both are considered to be exopolysaccharides the latter being more loosely adherent to the bacterial surface.[99] Virulence has been closely associated with encapsulation in the case of numerous gram-positive and gram-negative bacteria such as Streptococcus pneumoniae, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi and Neisseria meningitidis. Encapsulated bacteria have been found to be more resistant to phagocytosis than unencapsulated bacteria in almost all cases.[98] The capsule of E. coli has been shown to prevent the interaction of the phagocyte with subcapsular determinants which in the absence of the capsule allow ingestion of the organism.[98] Capsular antigens themselves are apparently unable to interact with the phagocyte to allow ingestion.

A second critical property of the E. coli capsule is that it does not fix complement by the alternate pathway.[98] Consequently, in the absence of specific anticapsular antibody, complement is not available on the surface of the organism to interact with the C_3 receptors on the phagocyte. In contrast, the lipopolysaccharide-rich outer

membrane of E. coli is capable of fixing complement via the alternate pathway. Anticapsular antibody is capable of rendering the bacterial agent susceptible to phagocytosis.[100] In some cases antibody directed against lipopolysaccharide O antigens may serve this function,[101,102] perhaps depending on the degree to which the O antigens protrude through the capsular material.

The capsules of other organisms apparently share the properties of E. coli capsules. The capsules of Staphylococcus aureus, Streptococcus pneumoniae and Cryptococcus neoformans have all been found not to fix complement by the alternate pathway.[103-105] It must be pointed out that the capsules of the above organisms did not block the fixation of complement to subcapsular surface components when incubated in normal serum. Apparently the capsule functions to block the access of phagocyte C_3 receptors to C_3 fixed on subcapsular surfaces. In addition, the capsule of C. neoformans has been postulated to block access of phagocyte Fc receptors to IgG on the subcapsular surface thereby preventing Fc-mediated phagocytosis.[106] Thus, it appears that capsular material is a very important virulence factor for microorganisms.

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SENSITIVITY OF SMOOTH *SALMONELLA CHOLERAE-SUIS* VAR *KUNZENDORF* FIELD STRAINS TO ANTIBODY COMPLEMENT UNDER VARIOUS CONDITIONS

SUMMARY

Seventy-two field strains of smooth Salmonella cholerae-suis var kunzendorf were obtained from outbreaks of swine paratyphoid. The field strains were examined for susceptibility to killing by antibody-complement (Ab-C) after they were exposed to Tris-EDTA, lysozyme, or saline solution. Considerable differences were found in susceptibility to killing by Ab-C in the strain population. Some strains were highly sensitive to Ab-C under all experimental conditions, whereas others became sensitive only after prior exposure to Tris-EDTA or to lysozyme. One strain was resistant to all treatments. Numerical decrease of colony-forming units by Tris-EDTA affected the population independently of decrease of the colony-forming units by Ab-C in saline solution. Evidence was obtained to indicate that Tris-EDTA may inhibit growth of S. cholerae-suis in addition to the documented effect of Tris-EDTA on the release of lipopolysaccharide from the cell wall of gram-negative bacteria.

INTRODUCTION

Salmonella cholerae-suis var kunzendorf is the etiologic agent of swine paratyphoid, a widespread septicemic disease of swine and distinguishable from other swine salmonelloses.[1,2] Salmonella cholerae-suis is also a major taxonomically and biologically distinct member of the genus Salmonella.[2]

The bactericidal actions of antibody (Ab) and of complement (C) are dependent on the character of the gram-negative bacterial cell surface,[3] the source of C,[3] the source and nature of Ab,[4] and the ionic environment.[5-7] Organic cations (such as tris[hydroxymethyl]amino-methane [Tris]), and surface active agents such as EDTA cause an increase of cell-wall permeability of smooth gram-negative bacteria, alterations of bacterial lipopolysaccharide (LPS) structure, and release of LPS into the environment.[6,8-10] Smooth salmonellae treated with Tris and EDTA were readily killed by Ab-C, and totally resistant to Ab-C killing in the presence of 0.002 M Mg^{2+}.[5]

The amount of LPS released from smooth gram-negative bacteria by Tris-EDTA equalled that obtainable by heating at 100°C for 1 hour[9] and was measured as 30% to 50% of total LPS.[8] Wardlaw[7] examined the lytic effects of numerous chemical and biological agents on a rough strain[a] of Escherichia coli under various ionic and pH conditions. Lysozyme

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[a]Eli Lilly and Company, Indianapolis, IN.

had no lytic activity by itself, but was active at low concentrations in the presence of EDTA.

Animal cells are lysed when incubated with Ab and C in the presence of Mg^{2+}. Gram-positive bacteria as a group and many smooth gram-negative bacteria are resistant to this effect when incubated in an ionic environment optimal for mammalian and avian cytolyses.[5,11] The ionic requirements for cytolysis of such smooth gram-negative bacteria thus constitute a significant departure from the cytolytic requirements of animal cells. It is not clear from the literature whether Tris and EDTA are bactericidal in the absence of Ab-C or whether they contribute to the Ab-C bacteriolysis by causing the release of LPS and by unmasking Ab-C target sites.

We have not found any reports describing the selective susceptibility of large numbers of field strains of gram-negative bacteria to Ab-C lysis. The foregoing reports dealt with small numbers of stable laboratory strains. We thought that susceptibility to Ab-C of recently isolated field strains of S. cholerae-suis var kunzendorf under various conditions might vary and might be a useful indication of virulence and smooth LPS content. We investigated differences in susceptibility related to the presence (or absence) of Tris-EDTA and of lysozyme. We report the frequency of such differences among field strains and probe into the mechanism of action of the Tris-EDTA effect by measuring DNA replication in Tris-EDTA-treated and control bacteria. The purpose in the present report is to describe the distribution of 72 field strains of S. cholerae-suis var

kunzendorf with respect to susceptibility to Ab-C in saline solution, in Tris-EDTA, and in lysozyme.

MATERIALS AND METHODS

Salmonella strains

Salmonella cholerae-suis var kunzendorf field isolates (n = 120) were obtained from the National Veterinary Services Laboratory, U.S. Department of Agriculture (SEA), and were identified as hydrogen sulfide-positive, monophasic (6,7:1,5). The cultures were examined for smoothness by colonial morphology on Tergitol-tetrazolium agar[a], spontaneous agglutinability after 1 hour's autoclaving in saline solution, and by the acriflavine agglutination test.[12] A total of 72 smooth S. cholerae-suis strains meeting the foregoing criteria were retained for the present study. These were frozen in multiple aliquots at -70°C. A separate aliquot was used for each test, and subcultures were avoided.

Antisera and complement

The antigen used for immunization of 2 sows consisted of formalin-killed pooled cultures of S. cholerae-suis strains No. 4, 42, 44, and 54. After adjustment of the concentrated bacterin suspension to density corresponding to McFarland tube No. 4, adjuvant (15% aluminium hydroxide) was added. The 2 sows were immunized with 5 ml of bacterin given IM at weekly intervals over a period of 8 weeks. Immunization with bacterin was followed by immunization with

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[a]Difco Laboratories, Detroit, MI.

approximately 10^9 live S. cholerae-suis strain No. 42 organisms given IM on 2 consecutive weekly intervals. Samples of venous blood (jugular vein) were obtained from the sows 2 weeks after the last immunizing inoculation. Serum No. 12 had O agglutination titer of 1:640 and H agglutination titer of 1:16,000, and was used throughout this work as source of Ab after heat inactivation at 56°C for 30 minutes. Complement of swine origin consisted of a pool of ten 8-week-old pig sera prepared from venous blood (jugular vein) collected after the pigs had been fasted for a day.

Antibody-complement bactericidal test

Overnight cultures of 72 S. cholerae-suis field strains in nutrient broth (NB) were standardized to optical density 0.2 at 550 nm in a spectronic 20 spectrophotometer. This standard bacterial suspension was decimally diluted to 10^{-6} as follows: all except the 10^{-4} dilutions were made in NB; the 10^{-4} dilution was made in 0.15 M saline solution; 0.1 M Tris (pH 7.2) + 100 ug of EDTA/L; or 10 mg of lysozyme/L in 0.15 M saline solution (Table 1). The bacterial suspension was held for 10 minutes before proceeding with further dilutions in NB. Complement was absorbed with heated, packed S. cholerae-suis at 4°C before use in the bacteriolytic test. Equal volumes of 0.25 ml of 10^{-6} dilution of bacteria, C (1:30 final dilution in NB), and Ab (1:8,000 final dilution in NB) were mixed and incubated for 5 hours in a shaker-water bath (37°C). After incubation, 10 ul drops of the reaction mixture were plated (in

triplicate) on Tergitol-tetrazolium agar. Colonies were counted after 14 and 18 hours' incubation. In control tubes, Ab and C were replaced by 0.5 ml of NB. Drops with colonies too numerous to count were coded at 200 colony-forming units (CFU) for statistical analysis. Only 18 Ab-C-susceptible field strains were tested with lysozyme. The effects of duration of Tris-EDTA exposure were assessed on a sample of 12 strains by exposure to Tris-EDTA for 10 minutes and 40 minutes.

[^{3}H]Thymidine incorporation

Fourteen strains having a marked numerical decrease of CFU after Tris-EDTA exposure were selected from the pool of 72 field strains. An additional 9 strains resistant to Tris-EDTA were evaluated. The strains were streaked on Tergitol-tetrazolium agar and incubated overnight at 37°C. Isolated smooth colonies were transferred to Tripticase soy broth (TSB) and incubated for 24 hours at 37°C. Each strain was diluted to 10^{-3} in both Tris and saline solution. These were incubated 10 minutes at room temperature before final dilution to 10^{-5} in TSB. Each diluted culture (1 ml) was dispensed (in triplicate) into 16 x 100 mm borosilicate tubes. Each tube was pulsed with 0.5 uCi of [^{3}H]thymidine[a] for 5 hours at 37°C with gentle mixing. The bacteria were prepared for liquid scintillation counting by precipitation with 6% trichloroacetic acid. Each sample was counted

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[a]New England Nuclear, North Billerica, MA.

for 8 minutes. Statistical evaluation was performed by analysis of variance on the mean of triplicate counts.

RESULTS

Effect of bactericidal treatments on field strains

Triplicate CFU counts on 72 field strains produced significant treatment differences (P 0.001) between treatment groups (Table 1). Antibody and C effectively decreased CFU counts of the strains in the presence of Tris-EDTA or lysozyme, but not in saline solution. Individual frequency distribution of field strains revealed considerable heterogeneity in the population (Table 2). Only treatment A (Tris-EDTA + Ab-C) reduced CFU counts consistently, with the exception of only 1 strain (No. 113). Treatment B (saline solution + Ab-C) was least effective, with 29 strains showing no effect. Treatment C (Tris-EDTA) affected the sample population in a heterogenous manner, with only 11 strains being unaffected by Tris-EDTA.

Plots were made of comparative treatment effects on the strain population. Treatment A (Tris-EDTA + Ab-C) affected the population independently of treatment B (saline solution + Ab-C), and the high susceptibility of most strains to treatment A was distributed over a wide range of treatment B effects (Fig. 1). Twenty-nine strains were unaffected by treatment B (saline solution + Ab-C). Comparison of treatment A (Tris-EDTA + Ab-C) with treatment C (Tris-EDTA) demonstrated a strong killing effect by Ab-C at all levels of Tris-EDTA sensitivity (Fig. 2). This plot also identified 1 strain totally resistant to both treatments, and 10 strains sensitive to both treatments in excess of 95%. Treatment C (Tris-EDTA) was also

independent of treatment B (saline solution + Ab-C), resulting in a random plot, an indication that the susceptibility of some strains to Ab-C in saline solution is unrelated to susceptibility of others to Tris-EDTA (Fig. 3). It is to be noted that 1 strain (No. 113) was unaffected (0 CFU reduction) by all treatments (Fig. 1, 2, and 3).

Effect of Tris-EDTA

The effect of duration of Tris-EDTA exposure and of subsequent incubation in NB was examined on a sample of 12 Tris-EDTA-resistant strains. The results indicate that the Tris-EDTA effect was dependent on duration of exposure and duration of subsequent incubation (Table 3). Cells recovered from the 10-minute Tris-EDTA exposure after 6 hours' incubation in NB, but CFU were significantly decreased after the 40-minute Tris-EDTA exposure. These data indicated that our initial classification of the population of 72 strains into Tris-EDTA "resistant" and "susceptible" is valid only for the selected 10-minute exposure and that all smooth strains might be affected by prolonged exposure to Tris-EDTA.

The effect of Tris-EDTA on S. cholerae-suis field strains also was evaluated, using [^{3}H]thymidine uptake. Fourteen strains which had been demonstrated to be sensitive to Tris-EDTA on the basis of colony counts and 9 strains determined to be resistant by the same method were studied. Table 4 presents the results of the tests. The mean CFU count for Tris-EDTA-treated S. cholerae-suis in the sensitive group was 49.2 $\pm$ 10.8, compared with a saline control CFU count of 200

(too numerous to count). The mean CFU count for the resistant group was 188 ± 7.7 for Tris-EDTA, and 189 ± 7.2 for the saline controls. When evaluated for incorporation of [^{3}H]thymidine, Tris-EDTA treatment of the sensitive group reduced uptake to 70.7 ± 7.8% of the saline control. Incorporation by the resistant group was reduced to 77.0 ± 7.0% of the saline control. Analysis of variance between the Tris-EDTA-sensitive and -resistant groups revealed no significant ($P = 0.5828$) differences.

Effect of lysozyme

The effect of lysozyme + Ab-C (treatment E) was examined, using 18 of the isolates of *S. cholerae-suis* sensitive to Tris-EDTA + Ab-C. Compared with the overall effect of various treatments on the total population of 72 strains, lysozyme + Ab-C was less effective than Tris-EDTA + Ab-C, but was more effective than all other treatments (Table 1). Pretreatment with lysozyme alone did not significantly alter CFU counts (Table 1).

DISCUSSION

It was long assumed that the Ab-C dependent killing of gram-negative bacteria required test conditions similar to those used in Ab-C lysis of animal cells.[13] It was later determined that LPS side chains of smooth gram-negative bacteria are cross linked by divalent cations (Mg^{2+} and Ca^{2+}) and that this cross linking prevents the interaction of activated C components with cell wall receptors.[5,6,10,11,14,15] Substitution of Mg^{2+} by univalent organic cations reversibly alters LPS side chains and results in the release of large quantities of LPS; the process is made irreversible in the presence of the strong chelating agent EDTA.[6,8-10,16]

In the present study, 72 recently isolated smooth field strains of S. cholerae-suis var kunzendorf were examined for in vitro susceptibility to Ab-C killing. The majority of these strains were highly resistant to killing by Ab-C when preincubated in saline solution, but became highly sensitive to Ab-C killing when preincubated in Tris-EDTA (Table 2). Preincubation in Tris-EDTA without subsequent Ab-C reduced CFU counts of many strains, but this effect occurred independently of treatment with Tris-EDTA and Ab-C (Fig. 2). It is likely that reduced CFU counts resulting from Tris-EDTA treatment for 10 minutes were due to cell wall damage, rather than actual killing of bacteria. The Tris-EDTA-resistant strains were able to recover from this effect when the subsequent incubation was extended to 6 hours, and partial recovery occurred even when the Tris-EDTA treatment was extended to 40 minutes (Table 3). Preincubation of

bacteria in Tris-EDTA resulted in 25% reduction in [^{3}H]thymidine incorporation over saline solution-preincubated bacteria. This does not agree completely with the results reported by Leive,[16] who observed that brief EDTA treatment resulted in an increased permeability of E. coli to actinomycin D, but little or no effect was observed on the viability, growth rate, or normal RNA and protein syntheses. Part of this discrepancy may be explained by the relatively greater sensitivity of the [^{3}H]thymidine method used here for detecting bacterial growth.

Immunity to salmonelloses may depend in part on humoral, and in part on cell-mediated mechanisms. It was shown in mice that early blood clearance of S. enteritidis depended on opsonizing and cytophilic antibodies, whereas the cell-mediated immune response provided the host with protection from replicating virulent bacteria within the reticuloendothelial cells.[17] Attenuated live-salmonella vaccines were found to be consistently more effective in conveying protective immunity than bacterins. Grouping of salmonellae on the basis of susceptibility to Tris-EDTA and to Ab-C may be an approach to selection of strains with various degrees of virulence. It may also be used as a measure of "smoothness," since the presently available methods are purely qualitative. Tris-EDTA was used for the measurement of released heat-stable antigens from Campylobacter fetus.[9] The release of LPS by Tris-EDTA was the source of immunizing antigen for the protection of pigs against enteric colibacillosis.[18,19] Classification of bacterial strains according to

susceptibility to chelators and to Ab-C may be an adjunct to the presently available methods for selection of potentially immunogenic strains of bacteria. The applicability of these methods would depend on the role played by cell wall components in pathogenesis and immunogenicity.

The work reported here also indicated that most smooth field strains of S. cholerae-suis var kunzendorf are susceptible to Ab-C lysis under optimal conditions of chelation. The relevance of this observation depends on the ionic environment encountered in vivo. This would also indicate that in vitro bactericidal tests may offer variable results depending on the ionic environment.

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Table 1. Effect of certain treatments on 72 field strains of S. cholerae-suis

Treatment	No. of CFU/10 ul
A - Tris-EDTA + Ab-C	16.8 ± 0.9
B - Saline solution + Ab-C	114.1 ± 1.4
C - Tris-EDTA	71.8 ± 1.9
D - Saline solution	184.0 ± 1.2
E - Lysozyme + Ab-C (18 strains only)	41.3 ± 8.3
F - Lysozyme (18 strains only)	166.3 ± 1.4

Data are expressed as mean of triplicate counts for each strain.

± 1 SEM.

Table 2. Frequency distribution of treatment effects on field strains of S. cholerae-suis

	Given categories of treatment effects*		
Treatment	0%	0≥80%	80%
Tris-EDTA + Ab-C	1†	10	61
Saline solution + Ab-C	29	32	11
Tris-EDTA	11	37	24

*Treatment effect (or percentage decrease of CFU count) = 100 - ($\bar{x}$ treatment/$\bar{x}$ control D) x 100.

† = 83.3; P > 0.005.

Table 3. Effect of duration of Tris-EDTA treatment and subsequent incubation on 12 field strains of S. cholerae-suis

Treatment	Given periods of incubation		
	0 Hr	4 Hr	6 Hr
Tris-EDTA 10 min.	0.2 ± 0.2	117.4 ± 15.2	191.7 ± 8.4
Tris-EDTA 40 min.	ND	9.9 ± 1.5	52.3 ± 7.2
Saline solution 10 min.	15.1 ± 3.3	143.3 ± 17.3	191.7 ± 8.3

Data are expressed as mean CFU count/10 ul (± SEM).

ND = not done.

Table 4. Comparison of [^{3}H]thymidine incorporation of Tris-EDTA-sensitive and -resistant strains of S. cholerae-suis var kunzendorf

Treatment	Mean CFU	Mean cpm
Tris-EDTA-sensitive strains (n = 14 strains)		
Tris-EDTA	49.2 ± 10.8	1,700
Saline control	200 ± 0	2,354
(percentage of control)*		70.7 ± 7.8
Tris-EDTA-resistant strains (n = 9 strains)		
Tris-EDTA	188.3	2,370
Saline control	189.3	3,339
(percentage of control)*		77.0 ± 7.0

*Equation: (cpm Tris-EDTA-treated bacteria/cpm saline treated bacteria) x 100.

cpm = counts per minute.

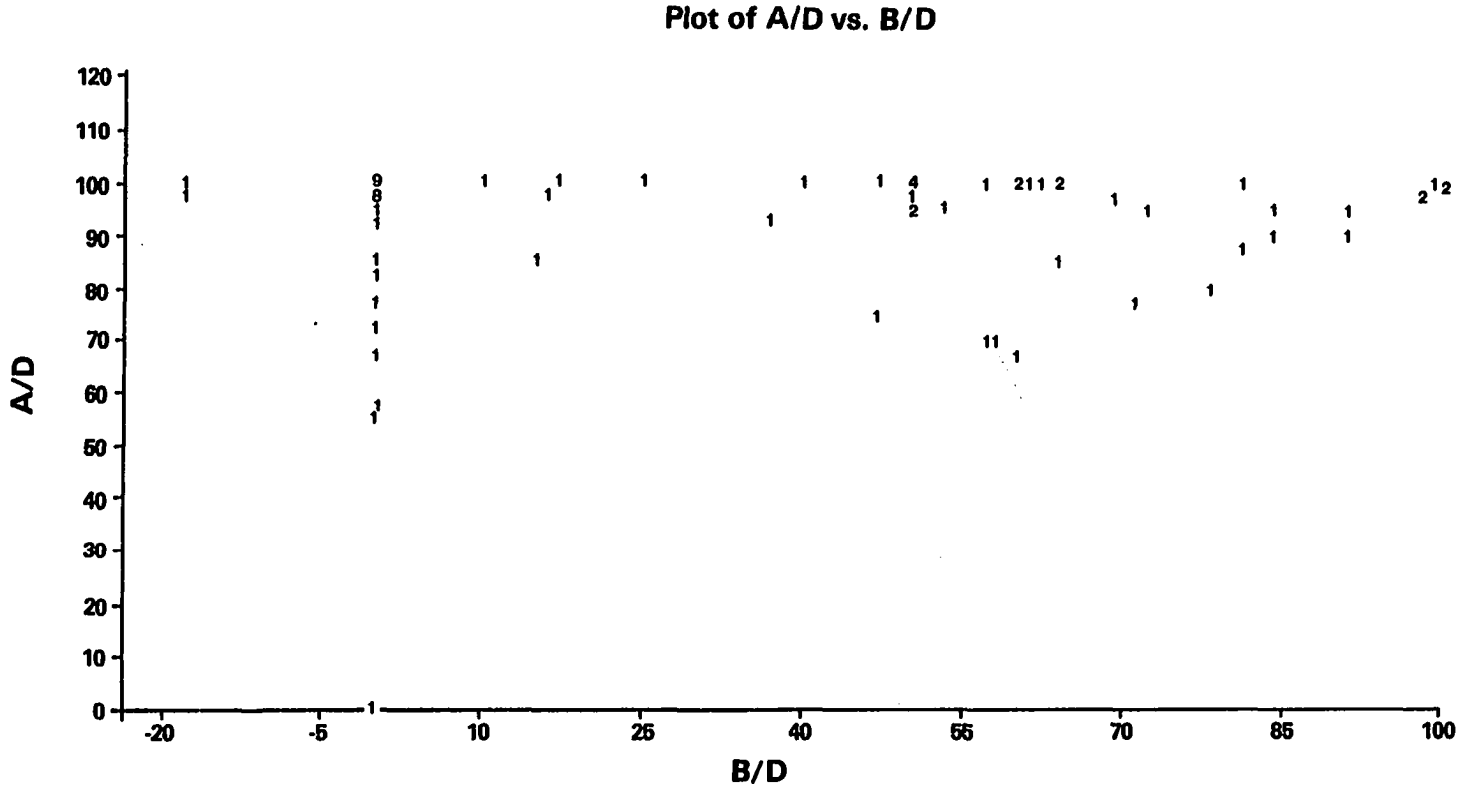

Figure 1. Distribution of 72 S. cholerae-suis strains based on susceptibility to Ab-C when preincubated in Tris-EDTA (treatment A) vs. susceptibility to Ab-C when preincubated in saline solution (treatment B). Coordinates - vertical = percentage of CFU reduction of treatment A over saline controls D; coordinates - horizontal = percentage of reduction of treatment B over saline controls D. Numerals indicate number of single digit observations.

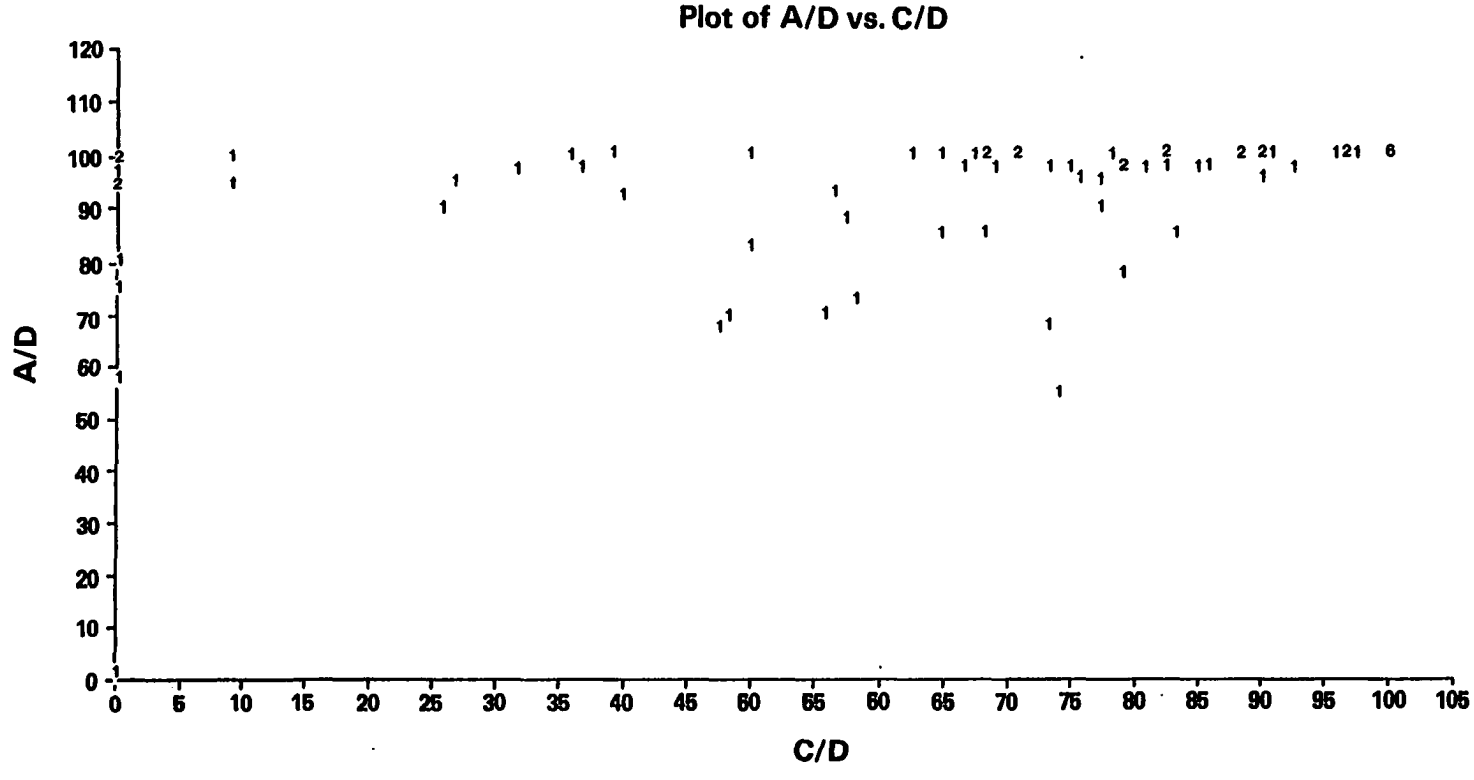

Figure 2. Distribution of 72 S. cholerae-suis strains based on susceptibility to Ab-C when preincubated in Tris-EDTA (treatment A) vs. susceptibility to Tris-EDTA without Ab-C (treatment C). Coordinates - vertical = percentage of CFU reduction of treatment A over saline controls D; coordinates - horizontal = percentage of CFU reduction of treatment C over saline controls D. Numerals indicate number of single digit observations.

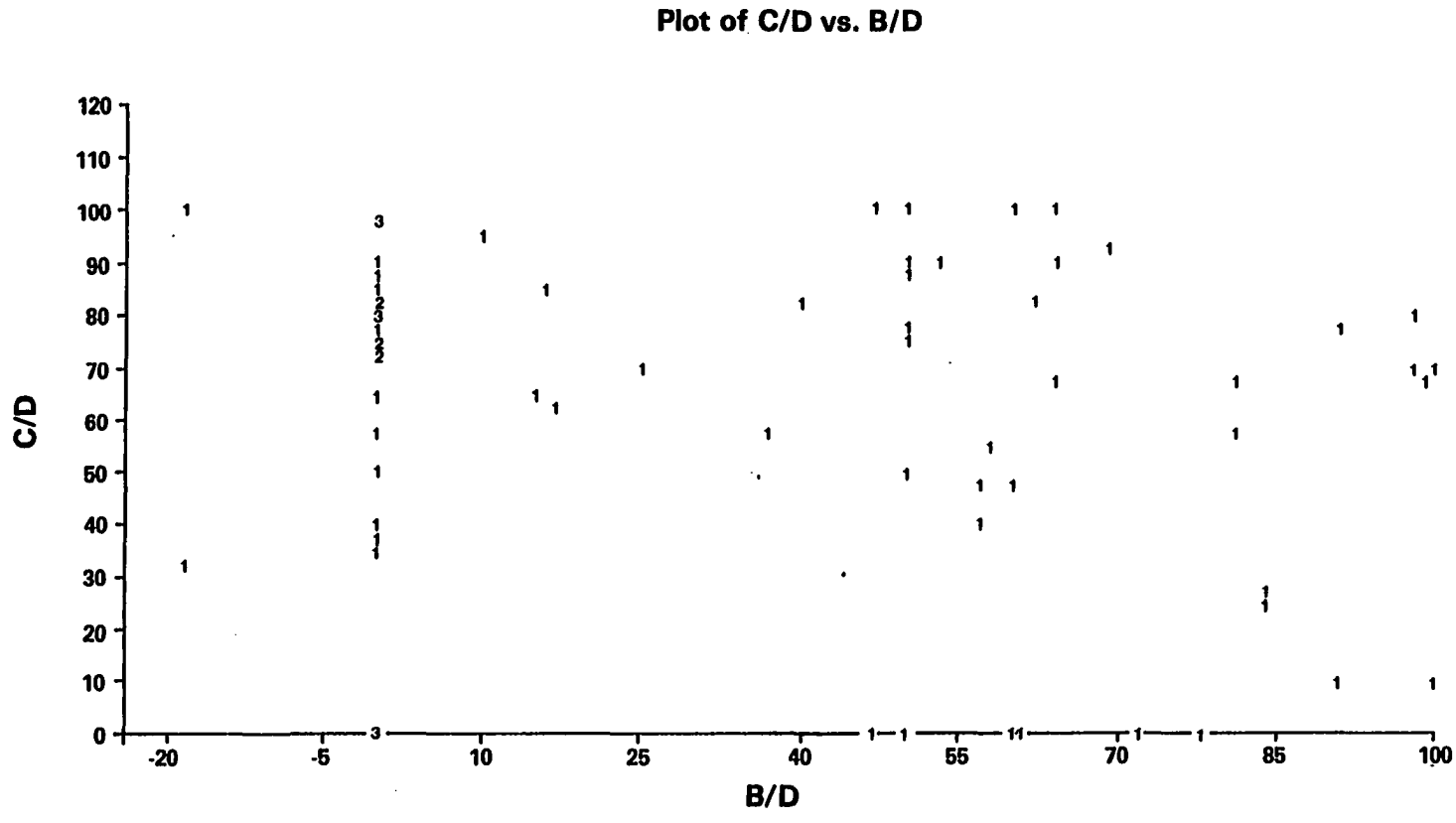

Figure 3. Distribution of 72 S. cholerae-suis strains based on susceptibility to Tris-EDTA (treatment C) vs. susceptibility to Ab-C when preincubated in saline solution (treatment B). Coordinates - vertical = percentage of CFU reduction of treatment C over saline controls D; coordinates - horizontal = percentage of CFU reduction of treatment B over saline controls D. Numerals indicate number of single digit observations.

SENSITIVITY OF SMOOTH *SALMONELLA CHOLERAE-SUIS* VAR *KUNZENDORF* TO KILLING BY PORCINE POLYMORPHONUCLEAR NEUTROPHILS AND ITS RELATION TO MOUSE VIRULENCE

SUMMARY

Twenty-two selected strains of Salmonella cholerae-suis var kunzendorf were evaluated for their in vitro susceptibility to the actions of porcine peripheral blood polymorphonuclear neutrophils (PMNs) and/or antibody (Ab). Strains were divided into 2 groups based on their previously reported sensitivity or resistance to antibody-complement (Ab-C) lysis. Strains resistant to Ab-C lysis resisted killing by PMNs to a greater extent than strains susceptible to Ab-C lysis. In the absence of PMNs, dilute Ab alone enhanced the growth of the Ab-C sensitive strains over that of the Ab-C resistant strains. Each of the strains was also injected intraperitoneally into a group of 5 mice. Four strains were totally avirulent for mice. Those mice challenged with the remaining 18 strains had a mean time to death of 5.1 $\pm$ 1.8 d. PMNs killed bacteria in both the virulent and avirulent groups but no significant differences were observed. Antibody alone had a stimulating effect on the growth of virulent strains but no effect on the avirulent strains. The difference between these groups was highly significant $P = .005$.

INTRODUCTION

Salmonella cholerae-suis is the primary etiologic agent of swine paratyphoid. The disease is commonly recognized to occur as two different syndromes, i.e., a peracute septicemic form most often observed in young weanling pigs and a chronic enterocolitis most often observed in feeder swine.[1,2] Investigation of host defense mechanisms against salmonellosis have generally been directed toward the roles of macrophages,[3,4] lymphocytes[5,6] or antibodies.[7] The role of PMNs in relation to host defense against salmonellosis has received less attention. Several recent reports have examined more closely the role of the PMN in resistance to this disease. Baskerville et al.[3] found that following intranasal challenge of pigs with S. cholerae-suis, all of the bacteria reaching the lung were quickly phagocytosed by pulmonary PMNs and macrophages. As observed by electron microscopy, many of the ingested organisms were killed but those that survived multiplied within the phagocytes. The PMNs were responsible for the majority of the early response to the challenge organism. Hsu and Radcliffe[8] stressed the significance of intracellular events in the early stages following phagocytosis. Smooth and rough strains of S. typhimurium were found to differ in the extent of intracellular killing rather than in subsequent multiplication. Smith et al.[9] correlated an observed increase in PMN bactericidal capability with an improvement in the clinical condition of swine infected 48 hr previously with S. typhimurium. Virulent S. typhimurium were found by Kossack et al.[10] to depress the burst of postphagocytic oxidative

metabolism in human PMNs when compared to avirulent strains. This indicates a possible mechanism for the selective intracellular survival of virulent organisms.

In earlier work,[11] we evaluated 72 field strains of S. choleraesuis for their sensitivity to antibody and complement bacteriolysis. The present work characterizes susceptibility of selected strains to phagocytosis and killing by pig peripheral blood PMNs. In addition, the relationship between the susceptibility of these strains and their virulence for mice is examined.

MATERIALS AND METHODS

Pigs

A group of 5 Yorkshire cross pigs weighing 50-70 lb was used throughout the experiment. One pig was bled per day. Bleedings were rotated so that each bacterial strain was tested against 3 pigs.

Bacteria

Twenty-two strains of Salmonella cholerae-suis var kunzendorf were selected from the pool of strains described previously.[11] The strains were selected on the basis of their sensitivity or resistance to lysis by Ab and C. Ten sensitive and 12 resistant strains were selected. Strains were maintained frozen at -70°C in sterile egg yolk. Aliquots were removed prior to each experiment and streaked on Tergitol-7 agar. The plates were incubated 24 hr at 37°C and single colonies transferred to trypticase soy broth (TSB). The broth cultures were incubated for 18 hr at 37°C. Immediately prior to use the cultures were adjusted spectrophotometrically to give an O.D. of 0.2 at 540 nm. These were then diluted 1:80 in M-199. The concentration of bacteria in the final reaction mixture was approximately 1×10^7 cfu/ml.

Antibody

Antibody was obtained from adult swine as described previously.[11] The final H titer was 1:32,000 and the O titer was 1:320. Dilutions given are based on final reaction mixtures. This antibody was

previously found to facilitate significant Ab-C bacteriolysis at dilutions of 1:10,000 or less.

PMNs

Blood from the anterior vena cava was collected in sterile ACD solution and PMNs were isolated using a modification of the method of Roth and Kaeberle.[12] The blood was centrifuged at 600 g for 15 min and the buffy coat and plasma discarded. The erythrocytes were flash lysed by adding 6 volumes of phosphate-buffered water (0.0132 M, pH 7.2) at 5°C. The mixture was restored to isotonicity after 60 sec. with 3 volumes of cold phosphate-buffered (0.0132 M, pH 7.2) 2.7% NaCl. The PMNs were sedimented by centrifugation at 500 g for 10 min, washed once in phosphate buffered saline (PBS 0.0132 M phosphate pH 7.2) and counted on a hemocytometer. Cell counts were corrected for eosinophils and mononuclear cells following a differential count of a Wright's stained smear. All preparations were corrected for viability as determined by trypan blue exclusion. PMNs were routinely 98-99% viable.

Medium

All PMN assays were performed in M-199[a] containing 10% newborn calf serum and buffered with 0.29% Na_2HCO_3 and 21 mM Hepes.

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[a]Grand Island Biologicals, Grand Island, NY.

Assay

The procedure is a modification of that of Foroosanfarr et al.[13] All assays were performed in triplicate in sterile disposable 16 x 100 mm borosilicate glass tubes. Final reaction volume was 1.05 ml. PMNs (2×10^6) were added to the appropriate tubes and incubated 15 min in a roller apparatus in an environment containing 5% CO_2 in air at 37°C. Antibody and bacteria (1×10^7 S. cholerae-suis) were added to appropriate tubes and the tubes incubated 30 min in a roller at 37°C in 5% CO_2 to allow for phagocytosis and killing. Tubes were then pulsed with 0.5uCi [^{3}H] thymidine.[a] The tubes were incubated for 3 hr in the roller apparatus at 37°C and 5% CO_2. The reaction was stopped with 2 ml cold 1 mM N-ethyl maleimide. Samples were prepared for liquid scintillation counting by precipitation with cold 6% trichloroacetic acid and solubilization with 0.5 ml Soluene 350.[b] Samples were counted for a minimum of 20,000 counts and corrected for both quenching and counts due to PMNs alone.

Experimental design

Control tubes containing PMNs but no bacteria were set up for each pig. These tubes were used to determine the background uptake of [^{3}H] thymidine by PMNs alone. All other tubes contained bacteria. The effects of various combinations of PMNs and/or Ab on each strain

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[a] New England Nuclear, Boston, MA.

[b] Packard Instrument Co., Downers Grove, IL.

of S. cholerae-suis were evaluated in tubes containing bacteria plus one of the following: 1) PMNs + 1:4000 Ab, 2) PMNs, 3) No PMNs or Ab, 4) 1:1000 Ab, 5) 1:4000 Ab and 6) 1:32,000 Ab. The mean CPM from each of the above treatments was adjusted for background counts due to PMNs alone. The adjusted mean CPM from the triplicate tubes of each of the 6 treatments was converted to log 2. Ratios were calculated between appropriate treatments for each strain on each day, e.g. log 2 treatment 1 - log 2 treatment 2. Means of these ratios were computed, converted to the antilog and multiplied by 100. Evaluation of statistical significance was by analysis of variance. Standard error of the mean (SEM) was computed from the mean of each strain.

Mouse challenge

Bacteria for mouse challenge were grown and standardized as described above. Following standardization, they were diluted in TSB to give a challenge dose of approximately 10^6 cfu in 0.2 ml. One group of five Sprague-Dawley mice[a] each weighing 20-25 g was inoculated intraperitoneally for each strain. The mice were examined twice daily for 30 days for mortality and the mean time to death (MTD) determined. Strains were grouped by MTD as either virulent or avirulent and the following three functions compared: 1) PMNs ÷ No PMNs or Ab, 2) 1:4000 Ab ÷ no PMNs or Ab, and 3) PMNs ÷ Ab.

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[a]Harlan Sprague Dawley, Indianapolis, IN.

RESULTS

Comparisons between the various treatments were performed on a daily basis for each strain to eliminate day to day variations. The means of these comparisons were converted to an integer by multiplying by 100.

The presence or absence of antibody was found to have an influence on the growth of S. cholerae-suis (Table 1). Strains incubated with a 1:1000 dilution of Ab had a mean[^{3}H]thymidine incorporation 1.28 times as great as the same strains incubated without Ab (Table 1). This effect decreased slightly (1.23 X) with an increased dilution of Ab to 1:4000. Decreasing the antibody to the 1:32,000 dilution resulted in the virtual loss of this effect (1.02 X). When the strains were grouped on the basis of their sensitivity to lysis by Ab-C (as determined earlier[11]) differences were observed between the sensitive and resistant groups. The differences achieved statistical significance ($P = 0.03$) only at the 1:4000 dilution of Ab. At this Ab level the strains most sensitive to Ab-C lysis grew better in the presence of Ab than did strains relatively resistant to Ab-C lysis. This was also true of the other two antibody dilutions, however, statistical significance was not achieved.

The effect of PMNs plus 1:4000 Ab on the growth of S. cholerae-suis was compared to the effect of PMNs alone (Table 2). When all strains were considered, PMNs + Ab reduced growth to a level 78% of that achieved with PMNs alone. No significant differences were observed between the Ab-C sensitive and resistant groups ($P = 0.4$).

The effect of PMNs plus 1:4000 Ab on S. cholerae-suis was compared with the effect of Ab alone. PMNs + Ab reduced the number of CPMs to 40% of the value obtained for Ab alone when all strains were considered. No significant differences were found between the Ab-C sensitive and Ab-C resistant groups. The values obtained were 38% and 42% respectively. PMNs alone reduced CPM's to 67% of Ab alone over all strains. Differences between the Ab-C-sensitive (mean = 55%) strains and Ab-C-resistant strains (mean = 80%) were highly significant P = 0.002. When the effect of PMNs + Ab on S. cholerae-suis was compared to the control tubes containing neither PMNs or Ab, CPMs were reduced to 49%. No differences were found between the Ab-C-sensitive (mean = 50%) and Ab-C resistant groups (mean = 47%). PMNs alone reduced CPMs to only 84% of control tubes containing neither PMNs or Ab. However, significant differences were observed between the Ab-C sensitive and Ab-C resistant groups P = 0.003. The values obtained for these were 74% and 95% respectively.

Intraperitoneal challenge of mice with S. cholerae-suis yielded variable results. Four strains were found to be avirulent for mice and the remaining 18 strains produced an MTD of 5.1 $\pm$ 1.8 days (standard deviation). The strains were grouped by MTD into virulent and avirulent categories and these two categories compared with the results of the in vitro assays (Table 3). PMNs alone reduced the CPMs to 64% and 76% of control tubes containing neither PMNs or Ab for the virulent and avirulent strains, respectively (Table 3). This difference was not statistically significant P = 0.20. When values

obtained with Ab alone were compared to those for control tubes containing neither PMNs or Ab a statistically significant difference was observed. The values were 128% and 100% for the virulent and avirulent strains, respectively. When PMNs alone were compared to Ab alone the virulent strains reduced CPMs to 56% of control versus 28% for the avirulent strains. This difference was not statistically significant $P = 0.19$.

Table 1. Effect of Ab on growth of S. cholerae-suis var kunzendorf. Mean ratio of Ab ÷ No Ab calculated in log base 2, converted to antilog and multiplied by 100 ± SEM. N = number of triplicate determinations. P > F calculated in base 2 log by analysis of variance.

Dilution of Ab	N	Overall Mean	Mean* Ab-C Sens	Mean** Ab-C Resist	P > F
1:1000	24	128 ± 7.8	137 ± 9.6	117 ± 11.7	0.2
1:4000	56	123 ± 3.9	132 ± 5.3	113 ± 4.9	0.03
1:32,000	24	102 ± 5.2	108 ± 4.5	93 ± 9.9	0.1

*Mean of 7 strains.
**Mean of 5 strains.

Table 2. Influence of pig PMNs and Ab on S. cholerae-suis var kunzendorf. Mean ratios calculated in log base 2, converted to antilog, and multiplied by 100 ± SEM. N = number of triplicate determinations. P > F calculated in log base 2 by analysis of variance.

	N	Over-All Mean	Mean Ab-C Sens	Mean Ab-C Resist	P > F
PMNs + Ab ÷ PMNs	49	78 ± 2.7	81 ± 1.9	76 ± 4.4	0.4
PMNs + Ab ÷ Ab	56	40 ± 4.7	38 ± 4.3	42 ± 7.0	0.8
PMNs ÷ Ab	49	67 ± 5.0	55 ± 4.0	80 ± 6.1	0.002
PMNs + Ab ÷ No PMNs or Ab	56	49 ± 3.6	50 ± 3.8	47 ± 5.5	0.9
PMNs ÷ No PMNs or Ab	49	84 ± 4.1	74 ± 4.7	95 ± 4.6	0.003

Table 3. Influence of pig PMNs or Ab on S. cholerae-suis strains grouped by mouse virulence. Mean ratios calculated in log base 2 converted to the antilog, and multiplied by 100. ± SEM N = number of strains. P > F calculated in log base 2 by analysis of variance.

	Mouse Virulent Strains N=18 MTD=5.1d	Mouse Avirulent Strains N=4 MTD=30d	P > F
PMNs ÷ No PMNs or Ab	64 ± 4.7	76 ± 6.2	0.20
Ab ÷ No PMNs or Ab	128 ± 4.2	100 ± 1.1	0.005
PMNs ÷ Ab	56 ± 5.7	28 ± 9.9	0.19

DISCUSSION

There is an increasing body of evidence that phagocytosis and killing of salmonellae by PMNs in the early stages of an infectious process may play a significant role in the eventual outcome of the disease process. This is especially evident when S. cholerae-suis is administered by an intranasal route resulting in the pulmonary form of salmonellosis. Baskerville et al.[3] found that the predominant response of the pig up to 4 days post challenge was mediated by the pulmonary PMNs. The inability of this PMN and pulmonary macrophage function to contain a virulent challenge gave rise to subsequent liberation of bacteria from degenerate phagocytes and eventual spread to other organs. The circulating PMNs could serve in an even more important fashion by limiting those organisms which enter the circulation via the usual enteric route. It is recognized that PMNs are more capable of antibacterial activity than macrophages in a non-activated state. Baskerville et al.[3] found that the mononuclear phagocyte was responsible for the dissemination of S. cholerae-suis to the loose connective tissue of the airways and the interalveolar septa of the lung. Friedberg and Shilo[14] found that the differences in susceptibility to killing among bacteria with different cell wall compositions was more pronounced when examined with PMNs. It would be of interest to study the interactions of pure preparations of PMNs with salmonellae rather than mixtures of PMNs and macrophages. The purity of the preparations in this study ranged between only 83 and 92 percent PMNs the remainder being eosinophils and mononuclear cells.

It is not surprising that significant differences were found between the ability of S. cholerae-suis strains to resist phagocytosis and killing. This variation is undoubtedly due in part to inherent differences in the composition and structure of the cell walls of various strains. Medearis et al.[15] demonstrated the importance of the O-specific side chains and polysaccharide core of E. coli in resistance to ingestion by PMNs in vitro. Friedberg and Shilo[14] examined wild type and cell wall mutants of Salmonella for sensitivity to ingestion and killing in vitro by mouse peritoneal macrophages and guinea pig PMNs. They demonstrated that a complete polysaccharide core of the cell wall is important for resisting ingestion and killing, and the presence of O-specific side chains contributes further resistance. UDP gal-4-epimeraseless mutants grown on galactose containing media were rendered phenotypically smooth and such bacteria were similar to wild type in resistance to ingestion and killing.

The role of the O antigen in the virulence of Salmonella has also been well demonstrated. Nakano and Saito[16] found that the number of O-specific side chains is important for the infectivity of S. typhimurium. Valtonen[17] studied the effects of O antigen variation on the virulence of S. typhimurium for mice. Via genetic manipulation he created strains of bacteria which varied only in their O antigen specificity. He found that significant differences existed in the virulence of such sister strains. These differences in virulence correlated with differences in the in vivo clearance of viable

organisms from the blood. His results point to phagocytosis as an important mechanism for determining the virulence of smooth Salmonella strains.

We have found that Ab-C sensitive strains of S. cholerae-suis were also more susceptible to killing by porcine PMNs compared to Ab-C resistant strains (Table 2). Surprisingly, the addition of antibody eliminated any statistically significant differences between these groups of strains. Dilute Ab alone had a marked effect on these strains and its presence may have negated the effect of the PMNs alone. This may have been due to agglutination of bacteria by the Ab dilution used with PMNs (1:4000), since Ab-C sensitive bacteria grew better in the presence of this concentration of Ab (Table 1). Since Ab alone is not bactericidal, and the requirements for opsonization were minimal in a 20:1 bacteria:PMN ratio, little difference was seen in PMN killing of opsonized vs non-opsonized bacteria (Table 2). Four strains having no virulence for mice were identified out of the 22 strains tested. These four strains were found to be unaffected by 1:4000 Ab alone (Table 3). Virulent strains grew better in the presence of 1:4000 Ab. The work presented here offers suggestive evidence that strains of bacteria can be evaluated for virulence properties based on susceptibility to PMN killing, and that this property is related to susceptibility to Ab-C. In addition, the differential effects of Ab on virulent versus totally avirulent strains generates further questions worthy of investigation.

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THE RELATIONSHIP BETWEEN THE ANTIBODY-COMPLEMENT SUSCEPTIBILITY OF SMOOTH *SALMONELLA CHOLERAE-SUIS* VAR *KUNZENDORF* STRAINS AND THEIR VIRULENCE FOR MICE AND PIGS

SUMMARY

Previous work[1,2] had characterized a large group of smooth strains of Salmonella cholerae-suis var kunzendorf for susceptibility to porcine antibody-complement (Ab-C), phagocytosis and killing by porcine peripheral blood polymorphonuclear neutrophils (PMN), and mouse virulence. In the present work a selected group of strains was further evaluated in mice and pigs. An Ab-C susceptible, mouse virulent strain was observed to multiply rapidly to high levels in the spleens of infected mice when compared to Ab-C susceptible, mouse avirulent strains. Pigs were infected intratracheally with 10^8 bacteria of one of eight strains, four of which were susceptible to Ab-C and four of which were resistant. Pig virulence correlated well with Ab-C susceptibility except for one strain (38) which was Ab-C susceptible but relatively virulent. Mouse virulence also correlated with pig virulence except for two strains (10,61) which were avirulent for mice but relatively virulent for pigs. Distinct differences existed between mouse and pig virulence and the relation of virulence to Ab-C susceptibility. The least virulent strain was found to protect pigs against the most virulent strain indicating that virulence factor(s) may not be associated with immunogenicity.

INTRODUCTION

Salmonella cholerae-suis is the etiologic agent of swine paratyphoid, a widespread septicemic disease of swine and distinguishable from other swine salmonelloses.[3,4] Salmonella cholerae-suis infection continues to be a serious worldwide disease. During the 1981-1982 USDA reporting year the average morbidity and mortality for outbreaks of swine paratyphoid were 16% and 4%, respectively.[5] The disease can also take a mild or subclinical form and lead to unthriftiness and decreased weight gain.

A number of workers have reported on the pathogenicity of various strains of S. cholerae-suis.[6-9] Unlike most other salmonelloses of domestic animals, disease due to S. cholerae-suis frequently becomes septicemic resulting in rapid death.[3,4] An understanding of the reasons for the relative efficiency of this organism in evading the immune mechanisms of the pig is of great importance. It has been shown that major differences exist between the in vivo blood clearance of serum sensitive and serum resistant organisms.[10] In the mouse, strains of Salmonella and E. coli that are sensitive to killing by Ab-C have usually been avirulent.[11-14] Susceptibility to killing by Ab and C and the phagocytosis of bacteria appear to be important in the initial stages of the elimination of gram negative bacteria from the systemic circulation.[15] In addition, it appears that the factors responsible for susceptibility to Ab-C are related to or identical with factors responsible for phagocytosis.[2] Roantree and Pappas[10] determined that factors other than serum sensitivity were responsible

for complete clearing of an organism from the blood once a septicemia was established. However, they attribute an important role to the serum bactericidal system in clearance especially at low concentrations of bacteria. In earlier work,[1] we described the characterization of 72 smooth field strains of S. cholerae-suis var kunzendorf for possible virulence-associated properties. These strains were evaluated for their susceptibility to in vitro killing by porcine antibody and porcine complement under various conditions. A few strains were found to be completely susceptible and others were completely resistant. In subsequent work,[2] it was determined that susceptibility to Ab-C correlated well with the susceptibility of these strains to killing by porcine peripheral blood polymorphonuclear leukocytes (PMNs). However, the virulence of a selected group of strains for mice was not well predicted by the in vitro results. We report here the virulence of a selected group of these strains for pigs and evaluate the multiplication and survival of three of these strains in the spleens of mice.

Very large inocula of S. cholerae-suis have traditionally been employed in attempts to establish an infection. Schoening et al.[8] were able to consistently produce mortality by feeding viscera from pigs dying of S. cholerae-suis and by injecting serum from infected pigs. They also utilized 10-25 ml of a 24 hr broth culture to produce disease. Biester et al.[6] were able to consistently produce disease in swine but only when the culture was recently isolated or passaged. Seghetti[9] had similar results with large inocula via the intravenous

and oral routes. However, on subsequent attempts to produce infection per os using "several" different strains of S. cholerae-suis, he failed to produce disease. There appears to be disagreement in the literature as to the ability S. cholerae-suis to produce experimental disease. Recent reports[16,17] have indicated that a decreased inoculum could be employed if given by an intranasal or aerosol route. We report here the ability of an intermediate dose given via the intratracheal route to produce disease.

MATERIALS AND METHODS

Mouse spleen counts

Sprague-Dawley mice (Harlan Sprague Dawley, Indianapolis IN) were used to evaluate growth curves of strains 9, 33 and 38 in the spleen. Bacteria for inoculation were grown in TSB and standardized spectrophotometrically as previously described.[2] Mice were inoculated intravenously with 10^4 to 10^5 bacteria and sacrificed at intervals on 1,3,5,7,9,11 and 15 days post infection. Four mice were sacrificed on each day for each strain. Individual mouse and spleen weights were recorded. Spleens were harvested, homogenized, and serial 10X dilutions made of the homogenates in saline. Each dilution was plated in quintuplicate by the method of Miles et al.[18] Colonies were counted of the appropriate dilutions.

Experimental design: Pigs

This work was divided into three parts. Experiment 1 was performed on 3 groups of 16 pigs each. Pigs were infected intratracheally with one of three strains of S. cholerae-suis and monitored for temperature responses, weight gains, serologic responses, clinical condition, gross and histopathological lesions, and presence of S. cholerae-suis in the tissues at necropsy. In experiment 2, five additional strains of S. cholerae-suis were inoculated intratracheally into groups of 3 to 4 pigs each. These pigs were monitored for temperature responses and rate of weight gain. In experiment 3, three groups of 5 pigs each were inoculated with

mouse-avirulent strain 33 via either the intramuscular, subcutaneous or intratracheal routes and monitored for temperature responses and rate of weight gain. These and a control group of 4 pigs were challenged with mouse-virulent strain 38, 21 days following the strain 33 inoculation. Temperature responses and rate of weight gain were again monitored.

Pigs

All pigs used in this experiment were Yorkshire-Hampshire crosses ranging in weight from 6.8 to 10 kg and 4-6 weeks in age. Groups of pigs were housed in isolation units with solid concrete floors and fed a pelleted ration. All groups were given a period of 1 to 2 weeks to adjust before experimental manipulation was performed.

Bacteria

Eight strains of Salmonella cholerae-suis var kunzendorf were selected for comparison. These were selected on the basis of their in vitro susceptibility to killing by antibody and complement (Ab-C) and their virulence for mice as previously reported.[1,2] Strains were stored frozen and grown as previously reported.[2] Cultures in trypticase soy broth were adjusted spectophotometrically to approximately 2×10^8 organisms per ml. Exact concentrations were enumerated by plate counts of serial dilutions.

Inoculation

All inoculations of pigs in experiments 1 and 2 were performed by the intratracheal route. Pigs were held in dorsal recumbency with the neck hyperextended. One ml of standardized broth culture was injected into the trachea approximately 2 cm caudally from the larynx. A swallowing reflex was usually noted upon injection indicating possible reflux of a portion of the inoculum into the oropharynx. In experiment 3, groups of 5 pigs each were "vaccinated" with strain 33 via either the intramuscular, subcutaneous, or intratracheal routes and challenged with strain 38 intratracheally. One group of 4 control pigs was challenged intratracheally with strain 38.

Temperatures and weights

Rectal temperatures were obtained daily. Weights were taken at weekly intervals beginning 1 week prior to challenge through 3 weeks post challenge. Temperatures and mean daily rates of gain were computed for each group $\pm$ standard deviation.

Serology

Pigs in experiment 1 were bled pre-infection, at weekly intervals and at necropsy. Sera were stored frozen until use. An indirect hemagglutination test and an antiglobulin test were performed in duplicate for each sample. These were performed in U bottomed

microtiter plates[a] using sheep red blood cells sensitized with phenol-water extracted S. cholerae-suis LPS. Indirect hemagglutination titers were read at 1 1/2 hr. Plates were then carefully washed 3 times in phosphate buffered saline (PBS 0.0132 M phosphate pH 7.2) containing 1% bovine serum albumin.[b] A 25 ul drop of a 1:100 dilution of rabbit anti-porcine IgM[c] in PBS was added to each well of the microtiter plate. The plates were agitated and the IgM titers read at 1 1/2 hr.

Postmortem

In experiment 1, two pigs were necropsied from each group starting on the first day a significant increase in rectal temperature was noted. Where possible, a pig with a high temperature was paired with one having a low temperature to avoid skewing the results. Pigs were necropsied in pairs during intervals from 2 to 22 days post infection (DPI).

Gross pathology

In experiment 1, gross pathological lesions were recorded for each pig at necropsy. An assessment of the general clinical state of the animal was also made.

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[a]Dynatech Laboratories, Alexandria, VA.

[b]Sigma Chemical Co., St. Louis, MO.

[c]Pel Freez Biologicals, Rogers, AR.

Histopathology

In experiment 1, sections of tissues were placed in 10% neutral buffered formalin during the necropsy. Where gross lesions were evident, an attempt was made to obtain a portion of the lesion along with adjacent normal tissue. The tissues were held in formalin for a minimum of 2 weeks before sectioning. Hematoxylin and eosin (H&E) stained sections were evaluated for each tissue. The identity of each section was masked and the slides graded for lesions a minimum of 3 times by the following criteria:

Lung Grade (1) = few or no lesions.

Grade (2) = interstitial infiltration by mononuclear cells with thickening of the alveolar septa and a low grade bronchopneumonia.

Grade (3) = moderate interstitial infiltration with mononuclear cells, diffuse multifocal necrosis with necrotic debris filling the surrounding bronchioles.

Grade (4) = severe bronchopneumonia with consolidation and necrosis.

Liver Grade (1) = few or no lesions.

Grade (2) = small accumulations of inflammatory cells primarily mononuclear in nature, slight activation of Kupffer cells. Lesions were minor and indicative of some type of reactive

process.

Grade (3) = diffuse multifocal mononuclear infiltration with little or no necrosis.

Grade (4) = characteristic diffuse multifocal coagulative necrosis with mononuclear infiltration. Highly suggestive of salmonellosis.

Intestines Grade (1) = low grade inflammatory response with infiltration of eosinophils, neutrophils, macrophages and lymphocytes. "Crypt abscesses" may or may not have been present.

Grade (2) = epithelial hyperplasia and focal necrosis in addition to inflammatory cell infiltration. A low grade inflammatory response was present in the submucosa.

Grade (3) = focal necrosis (erosion) of epithelial cells with focal ulceration. A high grade inflammatory response was present in the submucosa.

Grade (4) = atrophy of mucosa with multifocal erosive to ulcerative lesions.

Culture

Sections of tissues immediately adjacent to those used for histopathologic study were removed as aseptically as possible and

cultured for Salmonella cholerae-suis. Standard methods were employed using tetrathionate enrichment and brilliant green agar plates. The tissues cultured were: 1) larynx, 2) pre-scapular lymph node, 3) bronchial lymph node, 4) lung, 5) liver, 6) spleen, 7) mesenteric lymph node, 8) gall bladder, 9) heart blood, 10) terminal jejunum, 11) terminal ileum, 12) cecum, 13) terminal spiral colon.

RESULTS

Bacterial counts from mouse spleen cultures

The spleen weight and spleen weight/body weight ratios were found to vary markedly between individual mice and no significant differences were detected between mice receiving strains 9, 33 and 38. The total spleen bacterial counts are plotted in Figure 1. Counts did not differ between the avirulent strains (9 and 33) and the virulent strain (38) on day 1 post infection. On days 3, 5 and 7 total spleen counts were at least 10^3 higher for strain 38 than for strains 9 and 33. Mice inoculated with strain 38 did not survive beyond sampling day 7 even when the inoculum was lowered to 10^1 organisms.

Pigs

Strains 33, 38 and 61 S. cholerae-suis var kunzendorf were selected for detailed study in experiment 1. These strains were selected because of their strikingly different behavior in the in vitro Ab-C and mouse virulence assays. Strain 33 was completely susceptible to Ab-C and avirulent for mice. Strain 38 was susceptible to Ab-C but highly virulent for mice. Strain 61 was refractory to Ab-C and avirulent for mice.

Mean rectal temperatures of pigs infected with these three strains are given in Figure 2. The shaded area of the temperature curve was that above 40.5°C (104.9°F). Increases in temperature were obtained with all three strains. Strain 33 caused a relatively mild increase in mean rectal temperature when compared to strains 38 and

61. The onset of the response caused by strains 33 and 61 was delayed by about 24 hr. from that observed for strain 38. Temperature responses to strains 33 and 38 were quite uniform within the group but those to strain 61 were characterized by a few animals with extremely high temperatures on a prolonged basis, a few with normal temperatures and the majority with moderate increases from normal.

Average daily gains of the three groups of pigs in experiment 1 are also presented in Figure 2. A significant decrease in rate of gain was observed in pigs infected with strain 38. A decreased effect was found with strain 61 and no effect was observed in those pigs infected with strain 33.

Results of the indirect hemagglutination test on the sera from these three groups of pigs are given in Figure 3. Mean titers were relatively low for all three groups. Highest titers in pigs infected with strains 33 and 61 were observed at 2 weeks post infection. Strain 38 titers were highest at 3 weeks, however this should be interpreted with caution since only two pigs from each group remained at this time. Significant differences in titer were observed between strains 33 and 61 but neither was significantly different from strain 38.

The addition of a 1:100 dilution of rabbit anti-porcine IgM to the washed cells used for the indirect hemagglutination yielded the results presented in Figure 4. The sensitivity of this assay appears to be much greater. The relative shape of the curve for each group and its position compared to the other groups is approximately the

same as observed with the indirect hemagglutination test. Significant differences between titers for the three groups were only observed at 3 weeks post infection; however, this must again be looked at with caution due to the low numbers of pigs remaining at this stage. While the sensitivity of the antiglobulin test appears much greater than that of the indirect hemagglutination, there appears to be a concommitant decrease in specificity as manifested by the pre-infection titers.

Frequencies of gross pathological changes were summarized for each treatment group (Table 1). Emaciation was a prominent feature only of the group of pigs infected with strain 38. Only one pig in each of the other two groups was so affected. Grossly visible pulmonary changes were observed in all three groups but were most widespread in pigs infected with strains 33 and 61. Consolidation was present in these two groups at a rate of 50 and 40 percent respectively. Splenic enlargement was most consistently observed in strains 38 and 61 as were gross changes in the mesenteric lymph nodes.

Frequencies of histopathologic lesions in the lung, liver and intestinal sections from the three groups of pigs were tabulated (Table 2). Essentially no differences were observed among the lung lesions produced by the three strains. A substantial increase in the mean liver lesion severity was observed for strain 38 (as compared to the other two strains). Strain 38 also produced substantially more severe lesions in all 4 localizations of the intestinal tract when compared to the other two strains.

The rate of recovery of S. cholerae-suis at post mortem from the tissues of pigs infected with the three strains were summarized in Table 3. The rate of recovery was computed by dividing the total number of positive tissue isolations by the number of pigs necropsied during each of the three time periods 0 to 7, 8 to 13 and 15 to 22 days post infection. The number of pigs necropsied during each of these time periods varied from 4 to 6. The total number of tissues cultured was 13 as given in the methods section. As expected, the highest recovery rates occurred during the early portion of the experiment for each of the three strains. Strain 61 had a significantly higher recovery rate during days 0 to 7 while strain 38 had higher recovery rates during the 8-13 and 15 to 22 DPI periods. S. cholerae-suis was recovered from every pig up to and including 13 days post infection from one or more tissues. However, after day 13 in groups infected with strains 33 and 61, 4 of 6 and 3 of 6 pigs respectively were culturally negative for S. cholerae-suis.

In the second experiment an additional 5 strains of S. cholerae-suis were selected for evaluation. Of these strains two were susceptible (9 and 10) and three were resistant (7, 11 and 110) to Ab-C. Strains 9 and 10 were both avirulent for mice and the other three strains were highly virulent for mice.

Figure 5 presents the temperature response curves of the pigs infected with the 5 strains. All strains produced increases in mean rectal temperature. These responses were least severe in pigs infected with strains 9 and 10 and most severe in pigs infected with

strains 7, 11 and 110. Significant decreases in the rates of weight gain occurred only in the groups infected with strains 11 and 110.

Table 4 presents the comparison of the results obtained in experiments 1 and 2 with the previously reported susceptibility to Ab-C and mouse virulence. It was found that Ab-C susceptibility predicted the virulence of these strains for pigs with all but strain 38. This strain was virulent for both mice and pigs.

On the other hand, mouse virulence was also fairly successful in predicting pig virulence except for strain 61 which was avirulent for mice and moderately virulent for pigs. Strains 7 and 10 were also less accurately predicted by mouse virulence than by in vitro Ab-C susceptibility.

Experiment 3 was performed to evaluate the ability of the least virulent strain (33) to protect against the most virulent strain (38). Figure 6 presents the mean rectal temperature responses of pigs "vaccinated" with strain 33 by either the im, sc or intratracheal routes. Significant differences were not observed between these routes of inoculation and the data from the three groups are therefore combined. A moderate rise in mean rectal temperature was observed in the vaccinated group as expected; however, this increase was not statistically significant. The pigs were challenged with strain 38 along with a control group of 4 pigs at 21 days following strain 33 vaccination. A moderate increase in mean rectal temperature occurred in the vaccinated group compared to the control group. A highly significant difference was observed between this response and that of

the control group which had a marked and prolonged increase in mean rectal temperature.

Figure 7 gives the results of the analysis of the rates of weight gain for the vaccinated vs. control groups of pigs. Following vaccination with strain 33, no decrease in rate of gain was observed. Following challenge with strain 38 however, a decrease in rate of gain was observed for both the vaccinated and control groups. This decrease was significant for only the control group.

DISCUSSION

The immune response of pigs to S. cholerae-suis is undoubtably dependent on many factors, some related to the bacterium and some dependent on the pig and its environment. Based upon previous work,[10,15,19] it appeared that structural and biochemical variations within a large population of isolates would in part determine the virulence of these isolates. Some of these variations manifest themselves in the organism's susceptibility or resistance to Ab-C and/or phagocytosis.

Our initial work[1] on a large number of smooth field strains of S. cholerae-suis var kunzendorf indicated that the vast majority of these strains were refractory to the bactericidal effects of the porcine Ab-C system. This result was expected in light of the nature of the pool from which these isolates were drawn. Only smooth isolates were selected and the pool consisted primarily of strains recovered from suspected cases of porcine salmonellosis. This is also in agreement with the work of others[10,20] who found the majority of smooth gram negative bacteria to be resistant to the effects of Ab-C.

Within this large pool however, there existed a number of strains which were susceptible to the bactericidal effects of Ab-C under all in vitro conditions. There were also a number which were quantitatively much more refractory than the group as a whole. We believed this afforded an opportunity to evaluate the importance of the Ab-C system in the response of the pig to infection with S. cholerae-suis.

In addition, we previously had evaluated the susceptibility of a group of strains to the killing effects of porcine polymorphonuclear leukocytes.[2] Those strains most susceptible to killing by porcine Ab-C were also more susceptible to killing by porcine PMNs.

The virulence of this group of strains for mice was also of great interest to us. Mice are frequently used as an experimental animal for studying salmonellosis. It has been demonstrated[21-23] that mouse serum is not bactericidal *in vitro* pointing to a potential difference between mice and pigs in the mechanisms responsible for immunity to *Salmonella* sp. Susceptibility to Ab-C apparently is not the sole criterion for virulence in mice. Preliminary work in mice (unpublished data) indicated that Ab-C susceptible strains 9, 33 and 38 when injected intravenously were reduced to low numbers in the peripheral circulation within 1 hr. However, only strain 38 multiplied within the spleen (Fig. 1), indicating that the ultimate fate of the mouse at least as far as strain 38 is concerned lies not with the Ab-C system. This agrees with the observations of others.[13]

We have attempted here to delineate the relative ability of *in vitro* Ab-C susceptibility vs. mouse virulence to correctly predict the pig virulence of a particular strain of *S. cholerae-suis*. Based upon the data presented, we have attempted to categorize the eight strains as either virulent or avirulent for pigs as listed in Table 4. It is to be noted that those strains susceptible to *in vitro* Ab-C bacteriolysis were relatively avirulent for pigs with one exception, that of strain 38. Indeed, in this and other work,[24] strain 38

appears to be highly virulent. This indicates that virulence factors exist in addition to those associated with resistance or susceptibility to Ab-C killing. This agrees with other observations[15,19] that factors other than serum sensitivity are important in virulence. The nature of such virulence factors has received some attention[25,26] but largely remains unknown. The ability to multiply rapidly and to high levels within the host is of course a major criterion of virulence and some[13] suggest that it is the most important criterion. The rapid multiplication is thought to enable the organism to pre-empt the immune response. Also, the ability to compete for nutrients *in vivo* has been determined to be significant.[22,27] Attempts to use *in vitro* growth curves of *S. cholerae-suis* to evaluate differences between strains were unsuccessful.[24] Of those strains tested, growth curves were nearly identical for virulent and relatively avirulent strains. We have presented data that suggest that the pig virulence of a given strain of smooth *S. cholerae-suis* can be predicted by the *in vitro* Ab-C susceptibility of the strain.

The mouse virulence model also appears to be useful in predicting the pig virulence of selected strains of *S. cholerae-suis*. It did fail to adequately predict the virulence of strain 61. This observation is the reverse of strain 38. Strain 61 was refractory to Ab-C killing, avirulent for mice, and moderately virulent for pigs. This may indicate a qualitative or a quantitative difference between the mouse and the pig in the immune mechanisms responsible for

elimination of S. cholerae-suis. These factors are possibly related to the cell mediated aspect of the immune response and warrant further investigation.

Because of the major differences existing between strains 33 and 38, it was of interest to evaluate the ability of strain 33 to protect against a virulent challenge with strain 38. In our interpretation, strain 33 was found to be partially protective against strain 38 challenge. Thus, virulence factors associated with strain 38 may not be essential for an effective immune response. Our work indicates that the mechanistic basis of the immune response of the pig to S. cholerae-suis is complex and worthy of further investigation.

Table 1. Macroscopic lesions in pigs inoculated with 3 strains of *Salmonella cholerae-suis* var *kunzendorf*

		GROSS LESION					
						Mesenteric lymph node	
Strain	n	Emaciation	Icterus	Sublobular necrotizing bronchopneumonia	Splenic follicular hyperplasia	enlargement	subcapsular hemorrhage
38	16	4 (25)	9 (56)	4 (25)	7 (44)	9 (56)	4 (25)
33	16	1 (6)	1 (6)	8 (50)	3 (19)	5 (31)	0 (0)
61	15	1 (6)	0 (0)	6 (40)	12 (40)	2 (80)	2 (13)

n = number of pigs.

Values given are number of pigs with a given lesion for each strain of *Salmonella*.

Numbers in parentheses are percentages.

Table 2. Mean grade of tissue lesion in pigs infected intratracheally with strains of Salmonella cholerae-suis var kunzendorf evaluated by histologic examination

		TISSUE					
Strain	n	lung	liver	jejunum	ileocecal junction	cecum	colon
38	16	2.5	3.25	2.0	2.7	3.3	3.5
33	16	2.75	2.25	1.4	1.9	2.5	2.9
61	15	2.47	2.60	1.2	1.3	2.3	2.4

1 = no or subtle lesions.

2 = indication of some type of reactive process.

3 = moderate numbers or severity of lesions.

4 = severe or characteristic lesions.

See detailed explanation in text.

Table 3. Rate of recovery of Salmonella cholerae-suis var kunzendorf from tissues of pigs infected intratracheally

Strain	Days 0-7	Days 8-13	Days 15-22	Number of neg pigs Days 0-13	Number of neg pigs Days 15-22
38	4.2	7.5	3.0	0	0
33	4.5†	4.5	0.5	0	4
61	7.5	2.3	1.0	0	3

†Values obtained by dividing the total number of positive tissue isolations by the number of pigs necropsied during a given time period.

Number of pigs necropsied varied from 4 to 6 for each time period.

Number of tissues cultured from each pig = 13.

Table 4. Relationship of in vitro antibody-complement susceptibility and mouse virulence of selected strains of Salmonella cholerae-suis var kunzendorf to pig virulence

Strain	Sensitive to Ab-C	Mouse virulence	Pig virulence
9	+	-	-
33	+	-	-
10	+	-	±
38	+	+	+
7	-	+	±
11	-	+	+
61	-	-	+
110	-	+	+

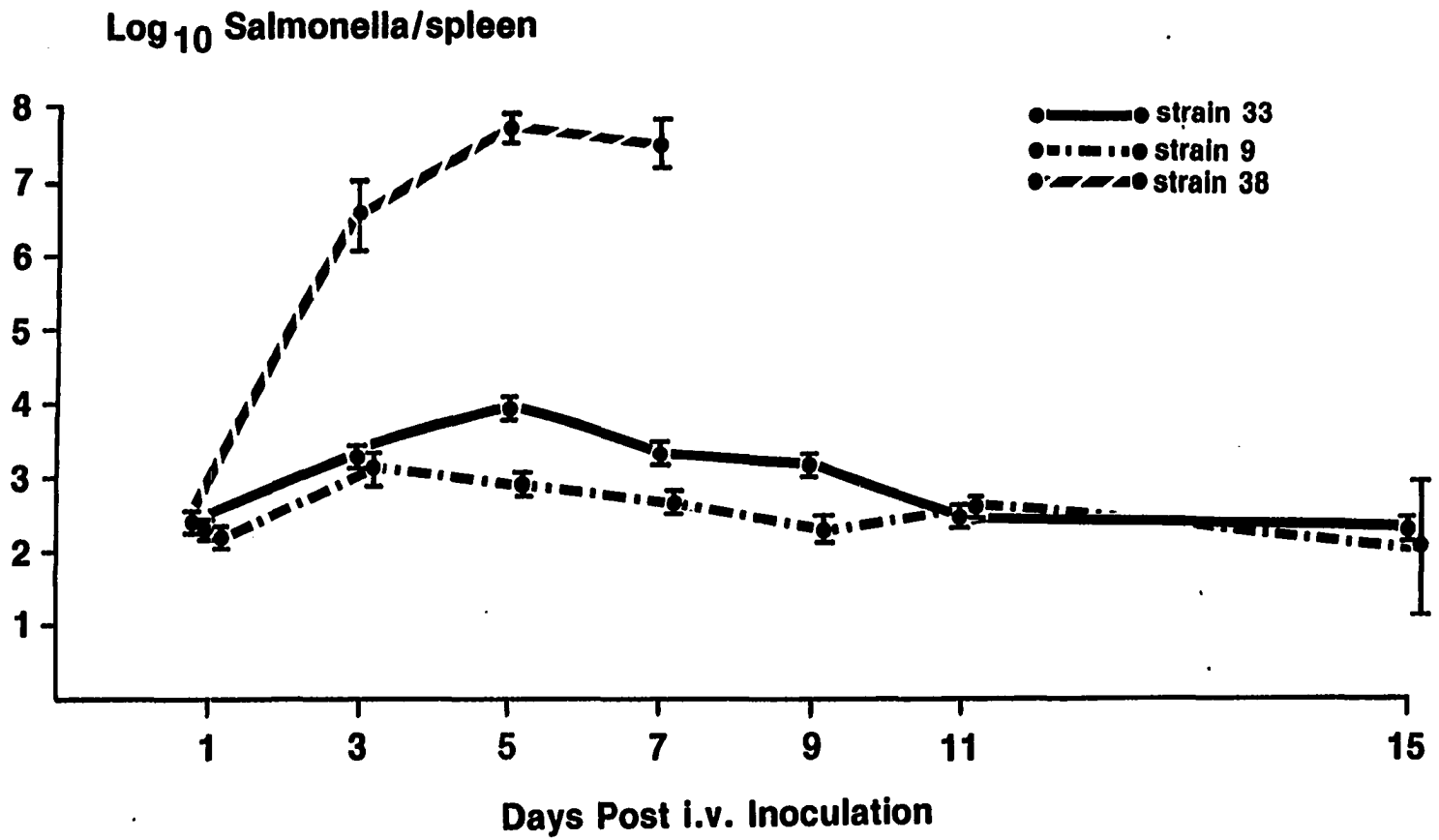

Figure 1. Splenic multiplication and/or clearance of 3 strains of S. cholerae-suis var kunzendorf from mice. Each point represents the mean of four mice ± SD.

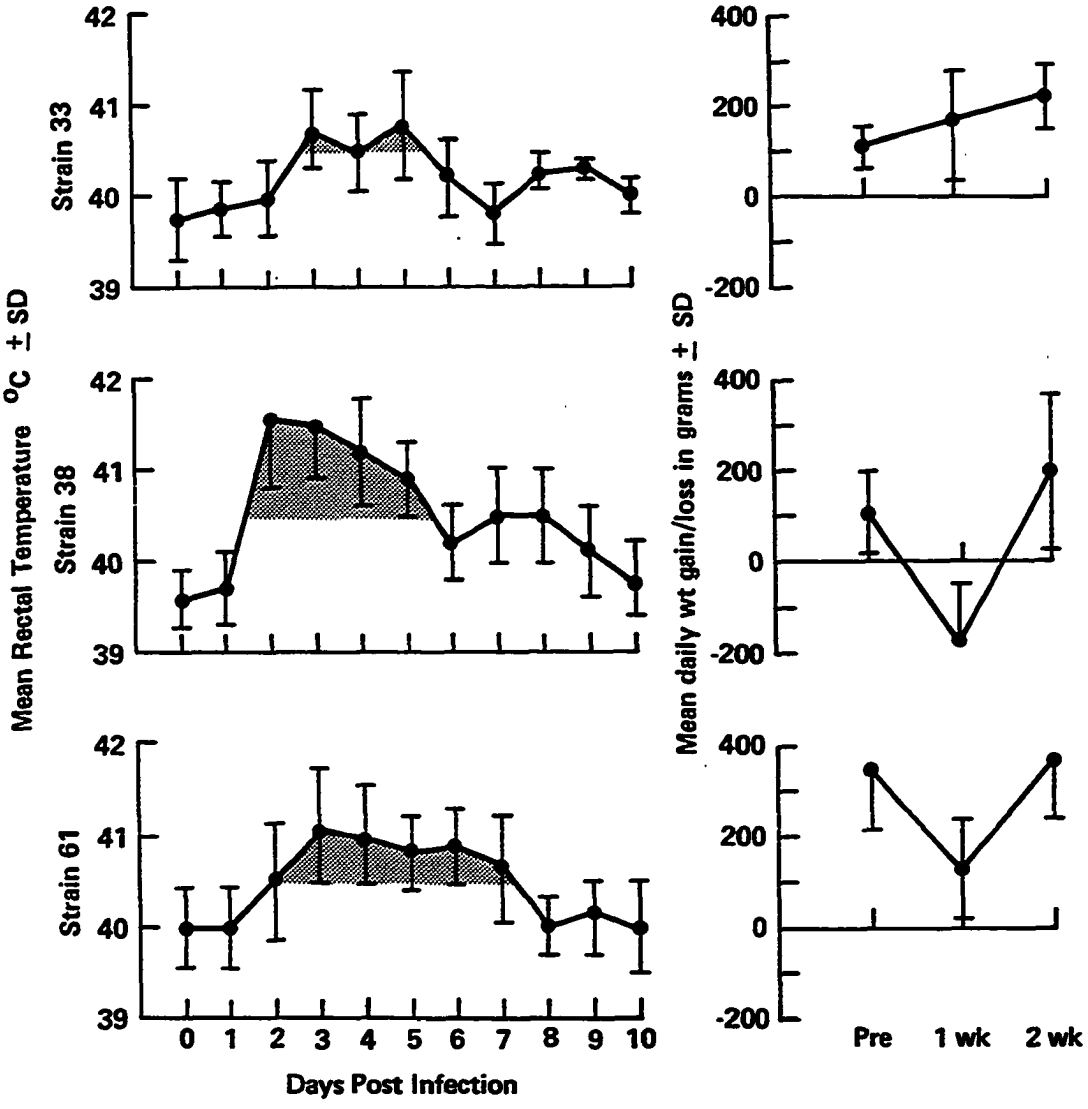

Figure 2. Mean rectal temperatures and mean daily weight gains or losses in pigs infected intratracheally with S. cholerae-suis var kunzendorf strains 33, 38 and 61. n = 16 pigs for each strain on day 0.

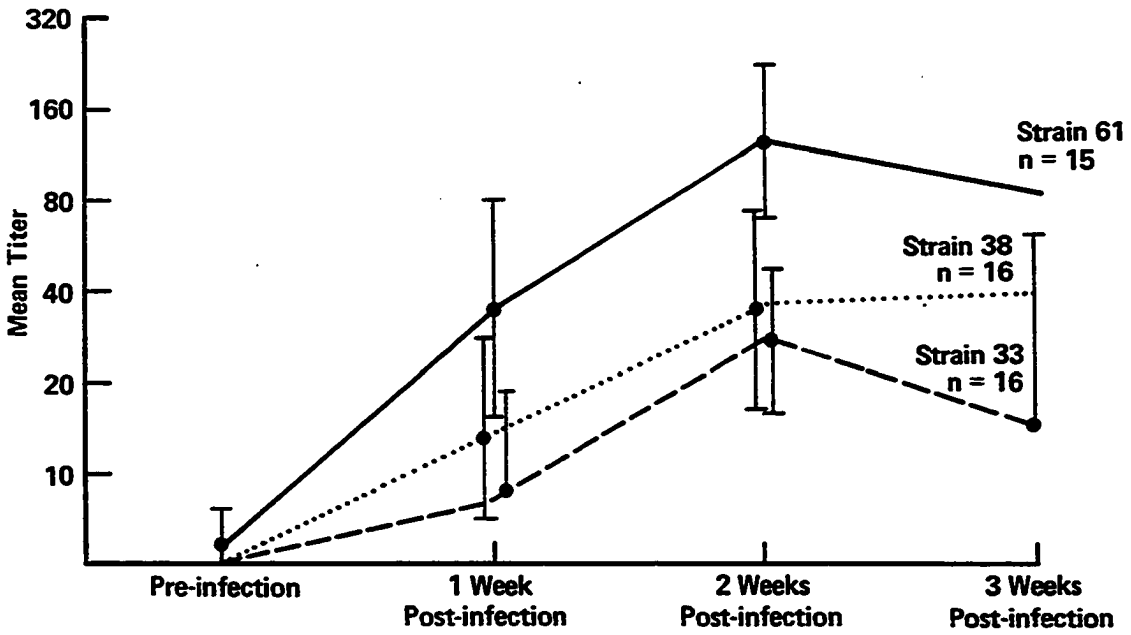

Figure 3. Mean indirect hemagglutination titers of sera from pigs infected with Salmonella cholerae-suis var kunzendorf. Titers are the reciprocal of the serum dilution ± SD. Sheep RBC's were sensitized with a phenol-water extract of S. cholerae-suis. Where no value is given for SD, all titers in that group of sera were equal. Number of sera varied from 16 at pre-infection to 2 at 3 weeks.

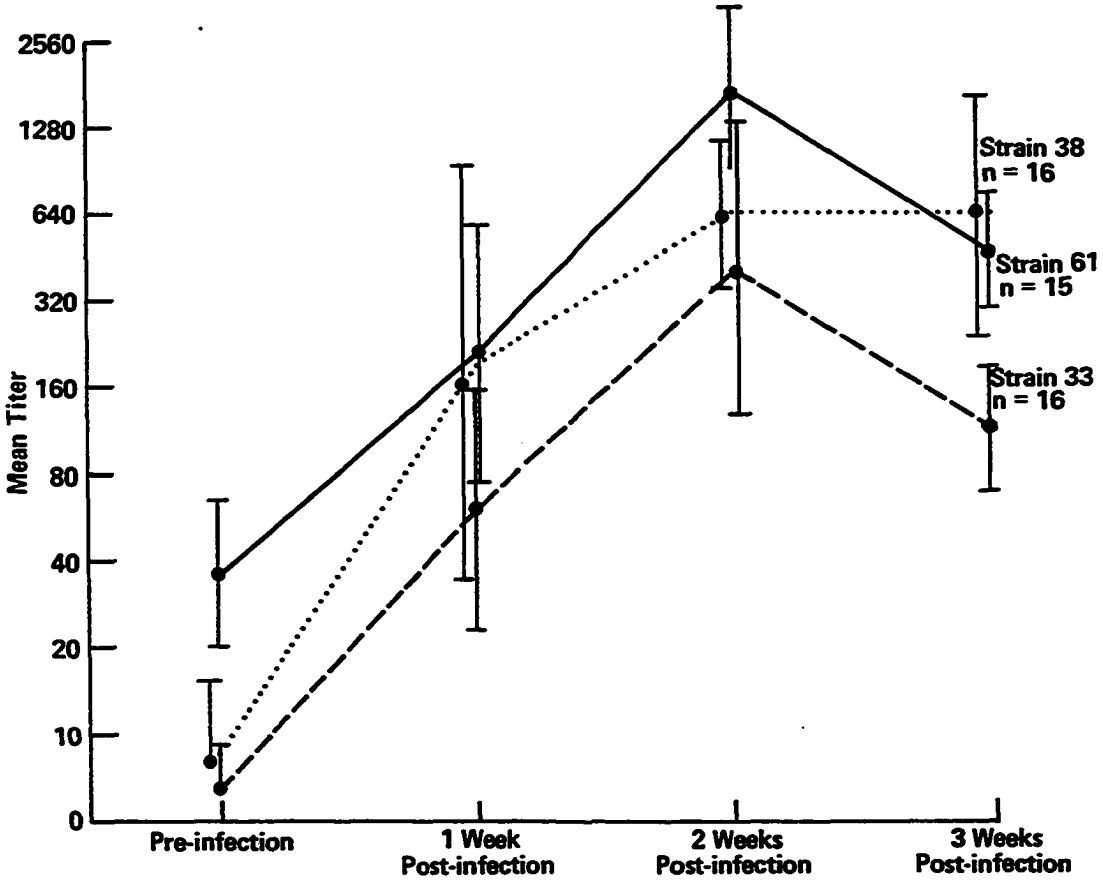

Figure 4. Mean antiglobulin titers of sera from pigs infected intratracheally with S. cholerae-suis var kunzendorf. Mean titers are the reciprocal of the serum dilution ± SD. Number of sera varied from 16 at pre-infection to 2 at 3 weeks post-infection.

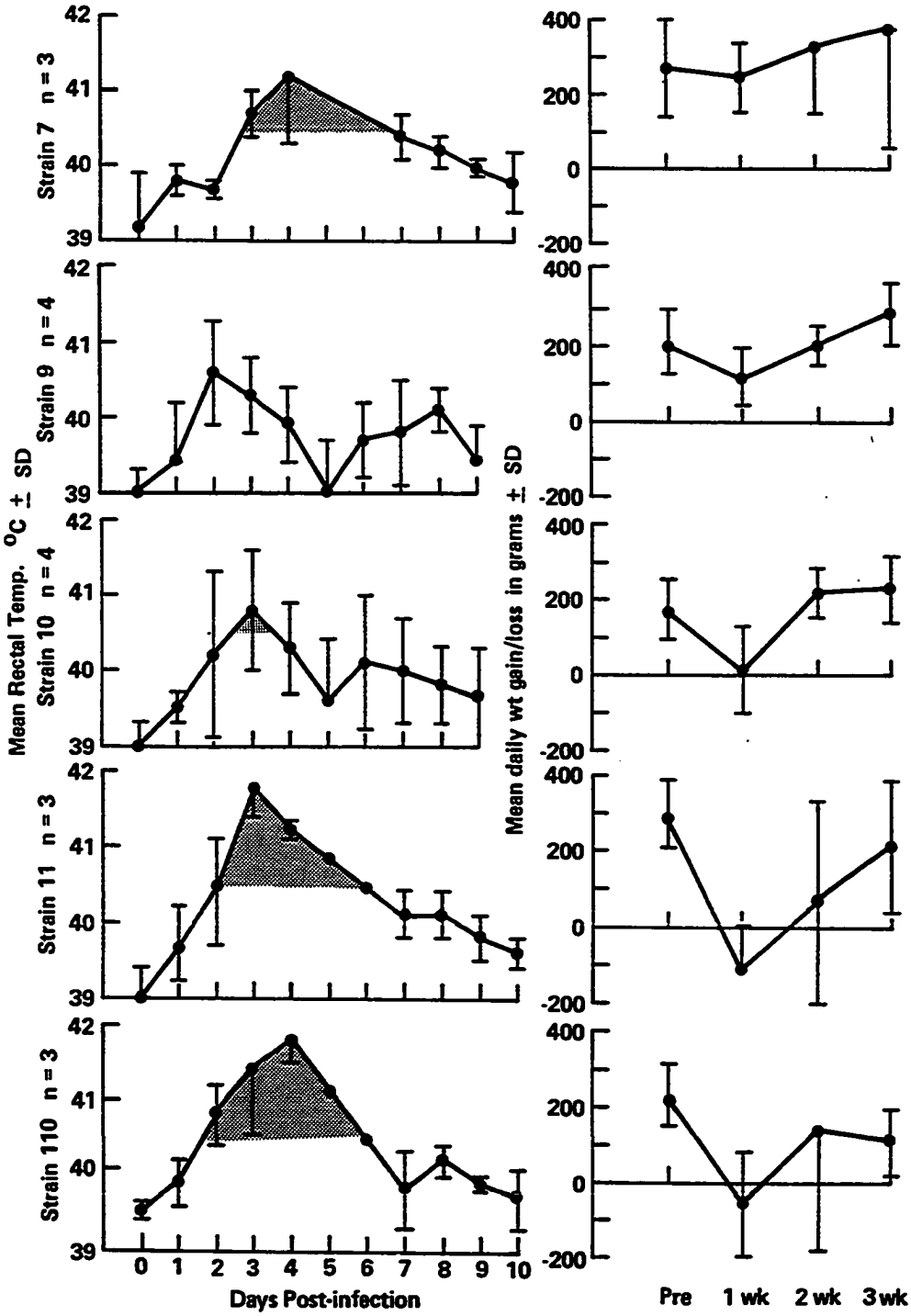

Figure 5. Mean rectal temperatures and mean daily weight gains or losses in pigs infected intratracheally with various strains of S. cholerae-suis var kunzendorf.

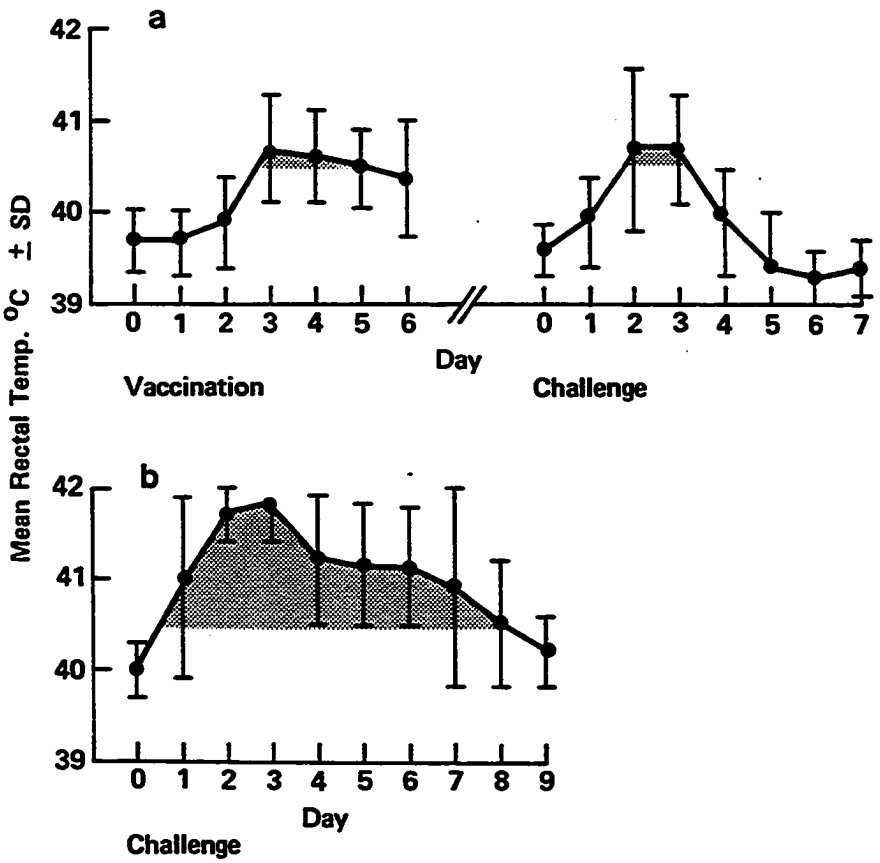

Figure 6. Mean rectal temperature response. a) Pigs inoculated with S. cholerae-suis strain 33 and subsequently challenged with strain 38 ± SD. n = 15 pigs. b) Control pigs challenged with strain 38. n = 4.

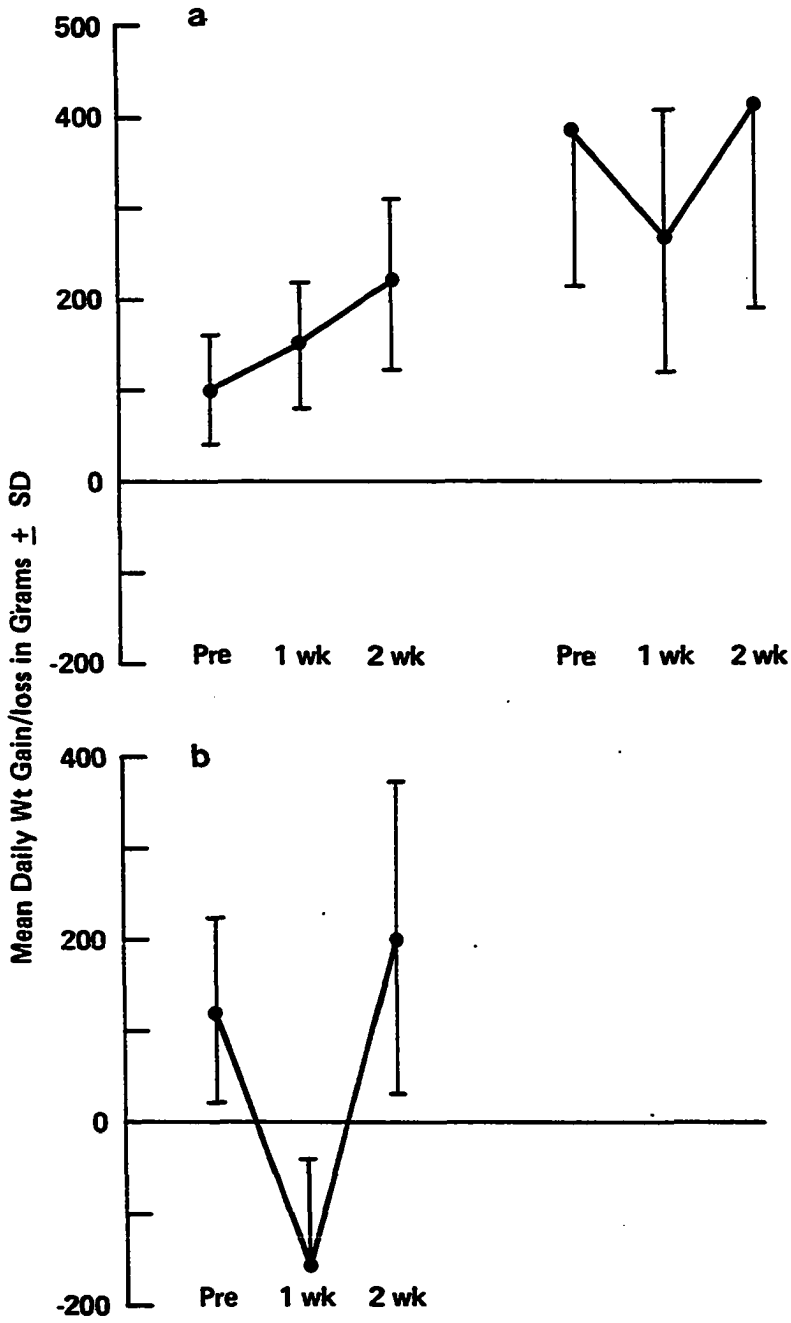

Figure 7. Mean daily weight gains or losses. a) Pigs vaccinated with S. cholerae-suis strain 33 and challenged with strain 38 ± SD. n = 15 pigs. b) Mean daily weight gains or losses of control pigs challenged with strain 38 ± SD. n = 4 pigs.

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THE EFFECTS OF CYCLOPHOSPHAMIDE ON THE IMMUNE RESPONSE OF PIGS TO *SALMONELLA CHOLERAE-SUIS* VAR *KUNZENDORF*

SUMMARY

The effects of the immunosuppressive agent cyclophosphamide (CY) on the immune response of the pigs to intramuscular challenge with a moderately virulent strain of Salmonella cholerae-suis var kunzendorf were examined. Five groups of Yorkshire-cross pigs weighing approximately 6 kg were given either Salmonella, CY or both at various intervals following initiation of CY therapy. CY was administered subcutaneously in 3 doses at 2-day intervals at a rate of 20 mg/kg body weight. CY was observed to produce significant depression of the numbers of circulating leukocytes particularly the polymorphonuclear neutrophils. Circulating lymphocyte numbers were reduced to 40-60% of normal values. Pigs given 3 x 10^6 S. cholerae-suis im at the time of the initial dose of CY were clinically very ill during days 6-12 and 2 of 5 died. In contrast, pigs given 3 x 10^6 S. cholerae-suis at the time of the third and last dose of CY did not become clinically ill until 10 days later. A significant rise in antibody titer to S. cholerae-suis was delayed in this latter group beyond that of the former and of pigs not given CY. A significant and prolonged increase in mean rectal temperature was observed in those pigs challenged at the time of the initial CY dose. Pigs were also sensitized to Mycobacterium avium 2 weeks prior to CY administration. Delayed hypersensitivity reactions were depressed in pigs receiving CY at the time of testing.

INTRODUCTION

Cyclophosphamide (CY) acts as a potent immunosuppressive agent in many animals and man.[1] The drug is converted by the liver into its active metabolites and exerts a nonspecific mitostatic action on a variety of rapidly dividing cell types. It has been well demonstrated that the major suppressive effects are on humoral immunity.[2,3,4] The numbers of both T cells and B cells are decreased but the effects on the macrophage were found to be minimal.[5] Others have found that a selective enhancement of the delayed hypersensitivity (DH) reaction occurs.[2,6,7] The effects of CY on the lymphoid tissue of the pig have previously been documented.[8,9] Mackie[9] determined that the major immunosuppressive effects of CY were exerted on the B-cell system in the pig and that T-cells were relatively unaffected.

The immune response to organisms of the Salmonella genus are thought to be primarily cellular in origin.[10] However, the humoral immune response seems to also play a role in limiting the infection.[11] Salmonella cholerae-suis infection in pigs is different from salmonelloses of other domestic animals. It has a relative propensity to rapidly become septicemic, occasionally leading to a fatal outcome.[12] In earlier work, we examined the role of antibody-complement (Ab-C) susceptibility of a large number of smooth strains of S. cholerae-suis,[13] and susceptibility to phagocytosis and killing by porcine peripheral blood polymorphonuclear neutrophils (PMNs) and found that the latter correlated well with Ab-C susceptibility.[14] Most strains susceptible to Ab-C and phagocytosis were relatively

avirulent for the pig. Those resistant to Ab-C and phagocytosis were in general relatively more virulent for the pig.[15] However, striking exceptions were found. The use of CY as an immunosuppressive agent afforded an opportunity to further evaluate the immune response of the pig to S. cholerae-suis.

The purpose of the present work was to examine the immunosuppressive effects of CY in pigs and the effects of this immunosuppression on infection of pigs with a selected strain of S. cholerae-suis. Strain 61 was selected for this work since it was moderately virulent for pigs and resistant to Ab-C and phagocytosis. In addition, the effects of CY on the delayed hypersensitivity response to a classic antigen (Mycobacterium avium) were also evaluated.

MATERIALS AND METHODS

Pigs

Pigs were Yorkshire crosses weighing approximately 6 kg at the initiation of the experiment. Thirty pigs were randomly divided into 6 groups of 5 pigs each and housed in isolation units with concrete floors.

Bacteria

Salmonella cholerae-suis var kunzendorf strain 61 was used as a challenge strain. The bacteria were stored frozen at -80°C until needed. An aliquot was streaked on a 5% bovine blood agar plate, incubated 18 hr. at 37°C and colonies picked to trypticase soy broth (TSB).[a] The broth culture was incubated 18 hr. at 37°C and adjusted spectrophotometrically. The bacterial suspension was diluted in TSB and 3.0×10^6 bacteria given intramuscularly.

Freeze-thaw antigen

Freeze-thaw antigen (FTA) was prepared from agar grown cultures of four strains of S. cholerae-suis var kunzendorf. The growth was washed from the agar with 0.85% sterile saline, washed 3 times in sterile saline, resuspended to 30 ml, and alternately frozen and thawed 20 times. The suspension was centrifuged and the supernatant passed through a 0.22 u filter. This material was stored frozen at -20°C until use.

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[a]Difco, Detroit, MI.

Cyclophosphamide

CY was diluted to 25 mg/ml in sterile saline just prior to administration, and injected subcutaneously at a rate of 20 mg/kg each on days 0, 2 and 4. This dose of CY was selected on the basis of data presented by Mackie.[16] Her work indicated good immunosuppression at this dose and significant mortality as the dose approached 50 mg/kg on a similar dosing schedule.

Temperatures

Daily rectal temperatures were obtained for each pig and a mean daily rectal temperature obtained for each group.

Total and differential white cell counts

A sample of blood in EDTA was obtained immediately prior to the initiation of the experiment and every second day thereafter. Total white blood cells (WBC) were counted with a coulter counter.[a] Blood smears were stained[b] and a differential count obtained for each.

Serology

A serum sample was obtained prior to the initiation of the experiment and at weekly intervals following injection of S. cholerae-suis.

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[a]Coulter Electronics, Inc., Hialeah, FL.

[b]Diff-Quik Harleco, American Scientific Products, Minneapolis, MN.

Sera were stored at -20°C and evaluated for antibody response by an indirect ELISA. Flat-bottom microtiter plates[a] were coated according to the method of Kennett et al. FTA was optimally diluted to 1:500 in 0.1M sodium carbonate, pH 9.6. One-hundred ul of this antigen dilution and 10 ul of a 1 mg/ml carbodiimide[b] solution in 0.1M sodium carbonate, pH 9.6 were added per well. Plates were incubated overnight at 4°C and washed 3X in PBS (pH 7.4). Ammonium chloride (0.1M, 100 ul/well) was added and the plates incubated on a shaker at room temperature for 30 minutes, followed by 3 washes in PBS. Two-fold dilutions of test sera were made in PBS-Tween (pH 7.5) and 100 ul of each dilution added to duplicate wells. Plates were incubated on a shaker as before, followed by 8 washes in PBS-Tween. Peroxidase-labeled rabbit anti-porcine immunoglobulin[c] (1:200 in PBS-Tween) was added (100 ul/well) and the plates incubated, and washed as before. Substrate (ABTS:2,2'-azino-di-3-ethylbenzthiazoline sulfonic acid[d] and H_2O_2 in 0.05M citric acid) were added (100 ul/well) and incubated at room temperature for 60 minutes. Optical density was plotted and a mean end-point titer determined for each group of pigs.

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[a] Dynatech Laboratories, Inc., South Windham, ME.

[b] "Cyanimide" Sigma Chemical Co., St. Louis, MO.

[c] Cappel Laboratories, Cochranville, PA.

[d] Sigma Chemical Co., St. Louis, MO.

Delayed hypersensitivity

Pigs were sensitized on arrival with 0.2 ml of Mycobacterium avium sensitinogen subcutaneously. A minimum of 2 weeks later, pigs were injected intradermally with approximately 0.05 ml M. avium PPD on the dorsal ear surface. Reactions were measured at 6, 24 and 48 hr. and a small portion of the DH reaction site removed for histopathologic examination.

Experimental

Pigs were divided randomly into 6 groups of 5 pigs each and treated as follows:

Table 1. Experimental design

Group	N	CY	M. avium Sensitized	Infected
1	5	-	-	+
2	5	-	+	-
3	5	+	+	-
4	5	+	+	+(Day 0)
5	5	+	+	+(Day 4)
6	5	-	+	+

Group 4 was infected with strain 61 at the time of the initial dose of CY. Group 5 was infected at the time of the final dose of CY. Day 0 is considered to be the first day of CY administration or the day of infection if CY was not given.

RESULTS

Cell counts

Dramatic decreases in mean WBC were recorded for pigs receiving CY (Figure 1). Mean WBC for all three groups fell to approximately 50% of pre-CY levels by day 2 and to 25% of pre-CY levels by day 4. Mean WBC remained low at 6 days and had returned to normal by day 10. Pigs receiving strain 61 im but no CY had a moderate increase in white cell numbers.

Neutrophils proved very sensitive to CY (Figure 2). Neutrophil numbers in pigs receiving CY (groups 3, 4 and 5) fell from approximately 6,000/mm^3 to 100-250/mm^3 by day 4. Neutrophils remaining at days 4 and 6 were hypersegmented. Normal neutrophil numbers were re-established by day 10. A dramatic rebound above normal occurred during days 10 to 16 in groups 4 and 5. After day 8, all pigs rebounded but those in a given group did not all respond on the same days, giving rise to large standard deviations. The return to normal neutrophil levels was relatively rapid. Mean neutrophil counts for group 4 peaked earlier than those of group 5. Group 4 was at normal levels (7,000/mm^3) by day 16 while the group 5 neutrophil count was at 35,000/mm^3 on this date. Pigs not receiving CY had a moderate but significant increase in neutrophil numbers probably as a result of the *S. cholerae-suis* infection (groups 1 and 6).

Lymphocyte numbers (Figure 3) decreased to 40-60% of normal by days 4 and 6 and returned to pre-CY levels by day 10 to 12. At the beginning of the study, pigs infected but not receiving *S. cholerae-*

suis (groups 1 and 6) had significantly higher lymphocyte counts than the groups receiving CY. Lymphocyte counts of groups 1 and 6 were still within published normal values at the outset. Lymphocyte numbers remained relatively steady in groups 1 and 6 throughout the experiment.

DH reactions

Pigs receiving CY (Groups 3, 4 and 5, Figure 4) had diminished DH responses to M. avium PPD when compared to the positive control groups (2 and 6). Group 4 which had received CY and S. cholerae-suis on day 0 had a DH reaction approximately the same as the unsensitized controls (group 1). Sensitization with M. avium sensitinogen was done 2 weeks prior to CY treatment, and the skin test therefore measured only the capability to respond to the cutaneous challenge, not the sensitization.

Rectal temperatures

Mean rectal temperatures of pigs infected but not receiving CY (group 1) were normal throughout the experiment (Figure 5). Pigs receiving only CY (group 3) had a slight decline in mean rectal temperature from days 0 to 4. On days 5 and 6, the mean rectal temperatures were elevated but then fell to normal levels by day 7. Pigs infected and given CY on day 0 (group 4) developed a temperature response which lasted from days 5 to 10. Pigs receiving CY on day 0 and S. cholerae-suis on day 4 (group 5) were not observed to have such

a dramatic response. This group attained a peak mean temperature of 40.5°C (104.9°F) on day 6 and thereafter declined.

Mortality

Three pigs died during the course of the experimentation (Table 2). One pig was in the group receiving only CY (group 3) and apparently died as a result of hemorrhage and swelling at the site of bleeding. Pigs given CY and infected on day 0 (group 4) were clinically quite ill from days 6 to 12. Blood was observed in their fecal material and diarrhea was present in all five pigs. One pig died on day 7. Postmortem analysis revealed hypoplastic mesenteric lymphoid tissue and pulmonary consolidation. Histopathologic examination of the lungs revealed a diffuse bronchopneumonia with infiltration by mononuclear cells and PMNs. The structural integrity of the mesenteric lymph nodes appeared to be lost and germinal centers were few in number and small in size. Multifocal coagulative necrosis was not present in the liver. Bacteriologic culture revealed S. cholerae-suis in the spleen only. Another pig in group 4 died on day 11. This pig also had hypoplastic mesenteric lymph nodes and pulmonary consolidation. Histopathologic examination of the mesenteric lymph nodes and pulmonary tissue revealed findings similar to those of the first pig that died from group 4. Diffuse multifocal coagulative necrosis was present in the liver of this pig. Bacteriologic culture revealed S. cholerae-suis in the lungs, liver, spleen, bronchial and mesenteric lymph nodes, gall bladder, jejunum,

ileocecal junction, cecum, and colon.

Serology

Pigs in groups 1, 4, 5 and 6 all attained equivalent endpoint titers with the ELISA test using FTA as the antigen (Table 3). The two control groups not given CY responded with a titer of 1280 at 2 weeks. Pigs given CY and infected on day 0 (group 4) had a mean titer of 640 by day 7 and 1280 by day 14. Pigs given CY and infected on day 4 (group 5) had a delayed antibody response but eventually attained a titer of 1280 by 21 days post infection.

DISCUSSION

The immune response of the pig to Salmonella cholerae-suis var kunzendorf appears to involve factors which are poorly understood. A large percentage of clinically normal swine harbor the organism[12] and yet relatively few ever exhibit severe clinical symptoms. The organism remains quiescent in the gut until stress or other factors allow or promote it to produce clinical disease. Infection also appears to be very age-dependent. Pigs over 10-12 weeks of age become relatively refractory to acute septicemia. A clear understanding of the immune response to this organism is important in understanding the pathogenesis of the disease syndrome. The immunosuppressant cyclophosphamide has been used in many other experimental situations to study various aspects of immune function. Mackie[9] indicated that the primary effect of CY on the immune system of the pig was directed at the B cell population. Given the broad range of mitostatic activity of CY, it seemed likely that significant effects on all rapidly dividing cell types was to be expected in the pig as has been reported for other species.

The depression of the circulating neutrophils and lymphocytes in this study appears to be similar to that observed by Mackie.[9] The neutrophils were particularly affected. Neutrophil counts at days 4 and 6 were approximately 100-250/mm^3. Those that were present appeared hypersegmented. Depression of lymphocyte counts were approximately 40-60%, roughly equivalent to those obtained by Mackie[9] at a dose of 30 mg/kg given three times.

It appears that in the pig a very narrow range occurs between a maximally suppressing dose of CY and a lethal dose. Administration of CY led to mortality in 1 of 5 pigs at the 20 mg/kg dose level. Had these pigs not been bled every second day this may not have occurred. Mackie[9] reported the development of abscesses and cellulitis at injection and bleeding sites as a common occurrence. For this reason, she administered antibiotics concurrently with the CY. The nature of the present work precluded antibiotic administration, however abscesses were not observed and reactions at the site of CY injection were self-limiting.

The depression of the antibody response at the 20 mg/kg level of CY appears to be incomplete (Table 3). Apparently, enough functional B cells remain in the early stages of the 4 day course of CY to initiate an Ab response in those pigs simultaneously given the first CY dose and S. cholerae-suis. These pigs were clinically ill on days 5-12 and remained stunted for the next two weeks. Immune function as measured by the white cell counts was recovering by day 7 or 8 and this was sufficient to avoid a fatal outcome in at least the 3 pigs which survived. Pigs in group 5 were given S. cholerae-suis at the point of maximal immune suppression. The Ab response was apparently delayed over that of the two control groups not given CY and the group infected concurrently with the initiation of CY treatment. Indeed, the latter group demonstrated the earliest formation of Ab under conditions which would appear to be least favorable to Ab production. In situ replication of the organism may have been favored in this

group giving rise to an increased antigen load. With larger inocula pigs have been demonstrated to produce Ab quite rapidly to the O antigens of S. cholerae-suis (within 4-5 days).[15] The data presented here tend to indicate that Ab is probably not the major factor determining the outcome of a S. cholerae-suis infection. Given the almost total depression of the neutrophils (Figure 2) it is also difficult to ascribe a significant role in S. cholerae-suis immunity to this cell population. It is possible that the initial inoculum of S. cholerae-suis was insufficient to produce disease without first multiplying to higher levels. To examine this possibility, group 2 (DH control group) was given CY subsequent to the end of the initial experiment. CY was given (20 mg/kg) on days 0, 2 and 4. The pigs were infected im on day 4 with a 100 fold higher dose (3×10^8) of S. cholerae-suis. The pigs became clinically ill but the symptoms were relatively mild and lasted only 3 days. It appears that an even larger infecting dose is required to consistently produce a fatal outcome under these conditions.

Two possible conclusions can be drawn from the above discussion: 1) immunity to S. cholerae-suis is dependent on factors other than Ab or neutrophils or 2) the dose of CY was insufficient or not prolonged enough to alter the B cell population and to suppress the neutrophils for a sufficiently lengthy period. The first conclusion points to the fixed macrophages as the major determinant of the outcome of S. cholerae-suis infection. Tripathy and Mackaness[5] have demonstrated the lack of an effect of CY on this cell population. This conclusion

agrees with the work of others that immunity to salmonella is primarily cell-mediated.[10] The second hypothesis may be hard to examine given the apparently narrow range between maximally suppressive and fatal doses of CY in the pig.[16]

Examination of the data on the DH response revealed suppression in pigs consistent with work in other species.[18,19] In cattle, CY pretreatment was found to enhance the DH response.[20] Also, timing of CY administration relative to sensitization has been reported to be very important in determining whether the DH reaction is suppressed or enhanced.[21,22] It appears therefore that under different conditions the DH reaction could have been enhanced rather than depressed as observed in this work.

Table 2. Mortality of pigs receiving cyclophosphamide and/or *Salmonella cholerae-suis* strain 61

Group	CY	Infected	Mortality
1	-	+	0/5
3	+	-	1/5
4	+	+	2/5
5	+	+	0/5
6	-	+	0/5

Table 3. Mean endpoint titers of pigs receiving cyclophosphamide and/or *Salmonella cholerae-suis* strain 61

		Endpoint Titer			
Group	n	1 week	2 week	3 week	4 week
1 & 6	10	0	1280	ND	1280
4	5	640	1280	ND	1280
5	5	0	320	1280	1280

n = number of pigs

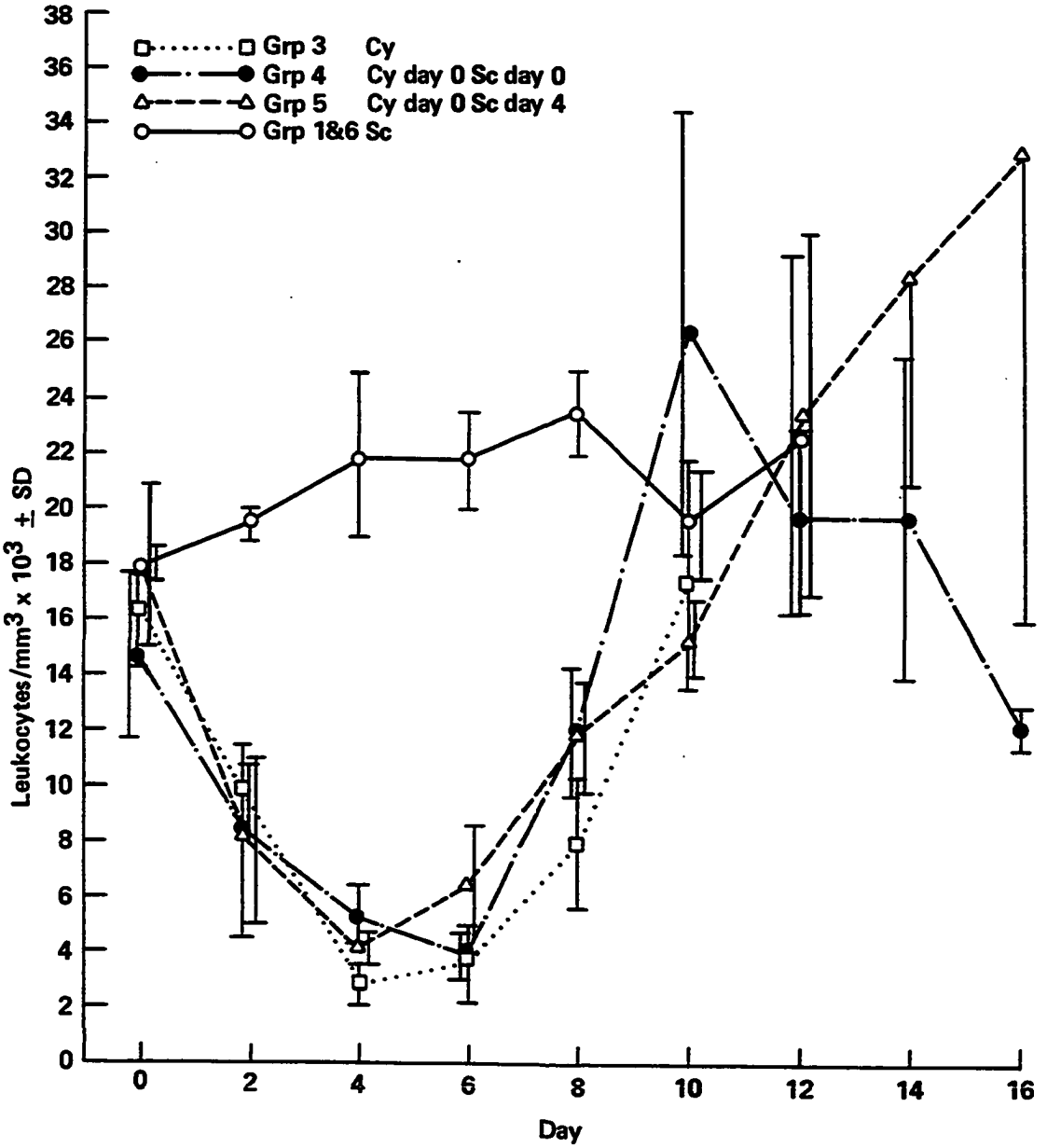

Figure 1. Mean leukocyte counts of pigs receiving cyclophosphamide and/or Salmonella cholerae-suis strain 61. Brackets indicate SD. n = 5 pigs/group.

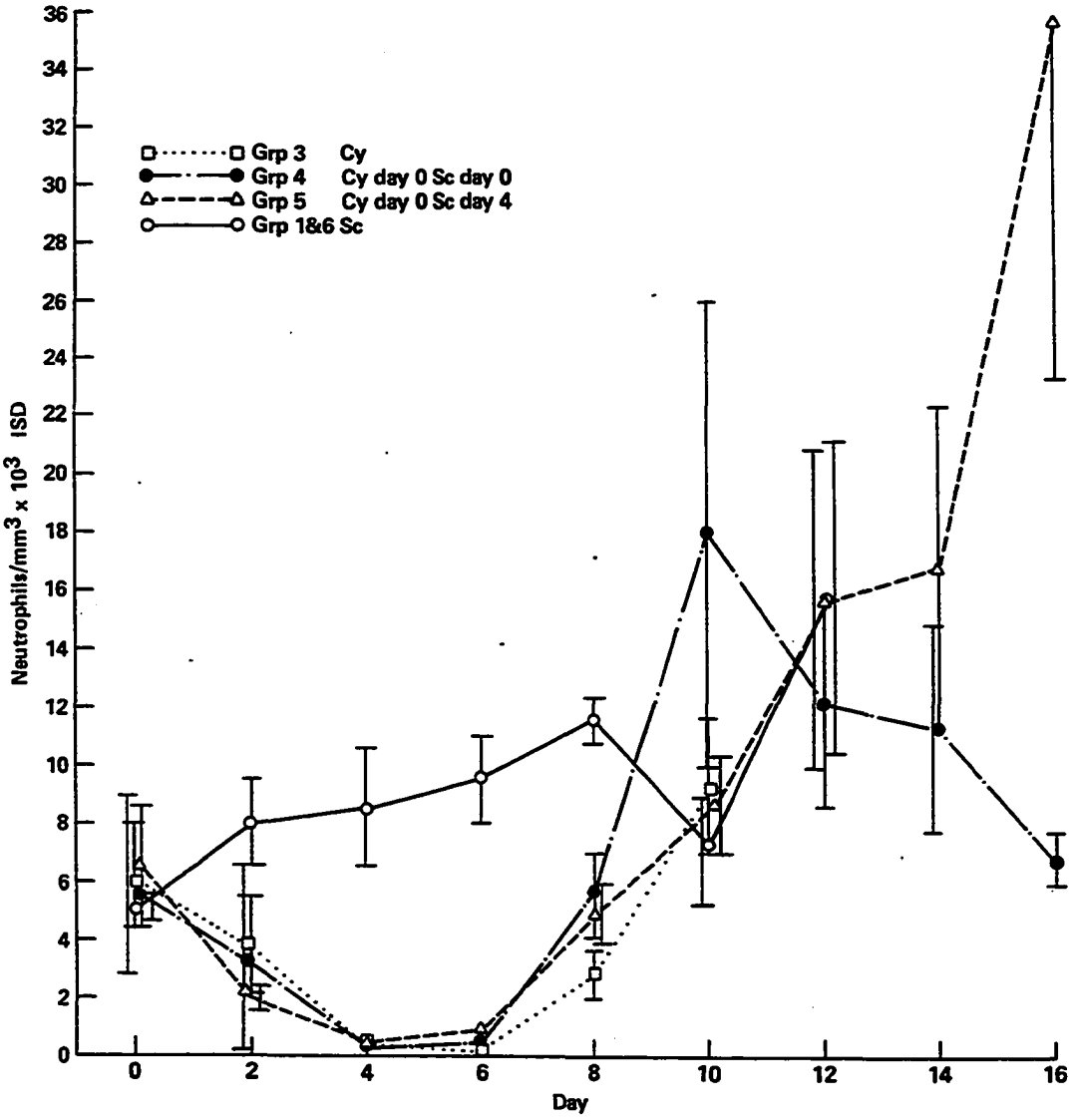

Figure 2. Mean neutrophil counts of pigs receiving cyclophosphamide and/or Salmonella cholerae-suis strain 61.

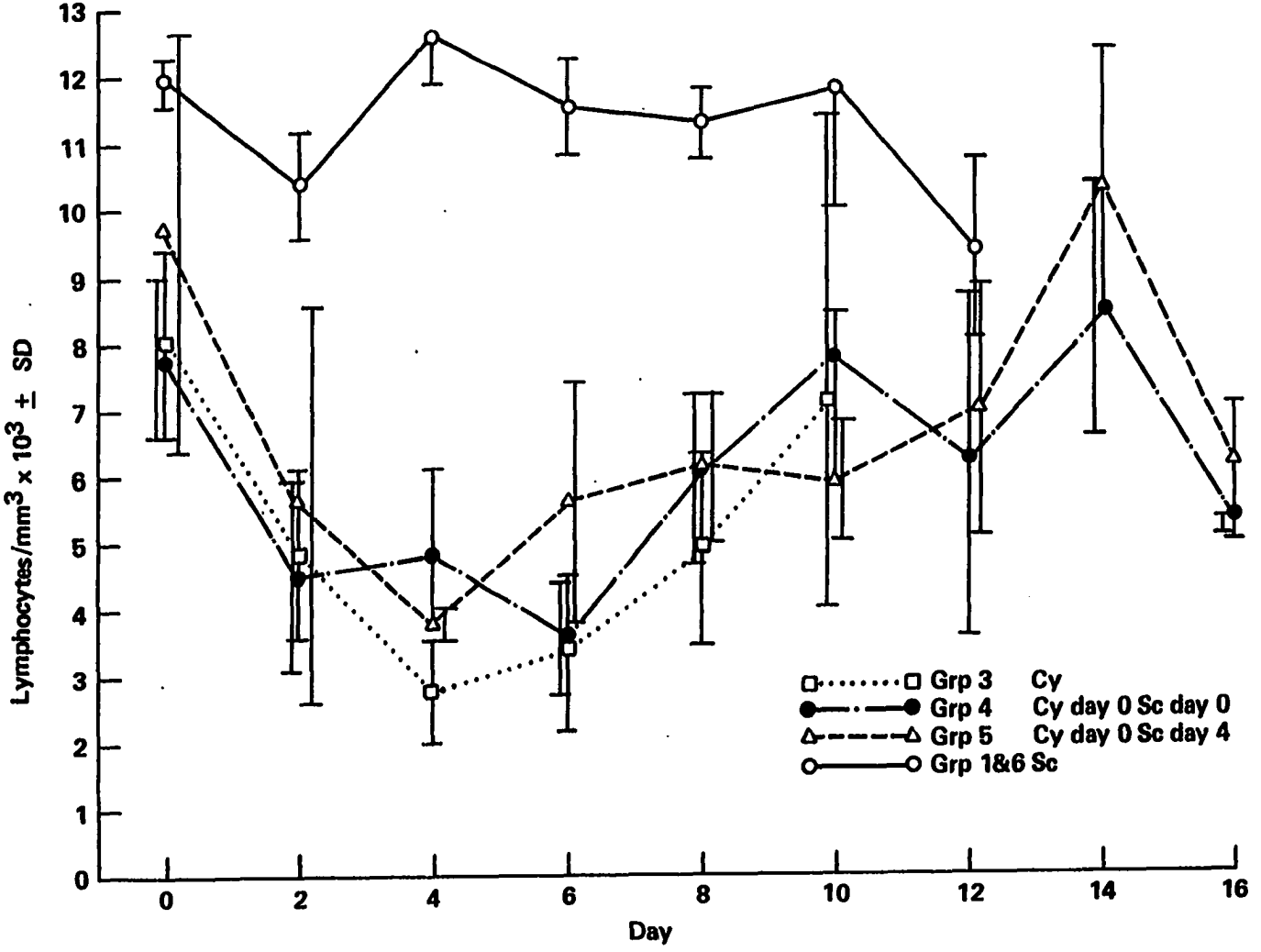

Figure 3. Mean lymphocyte counts of pigs receiving cyclophosphamide and/or Salmonella choleraesuis strain 61.

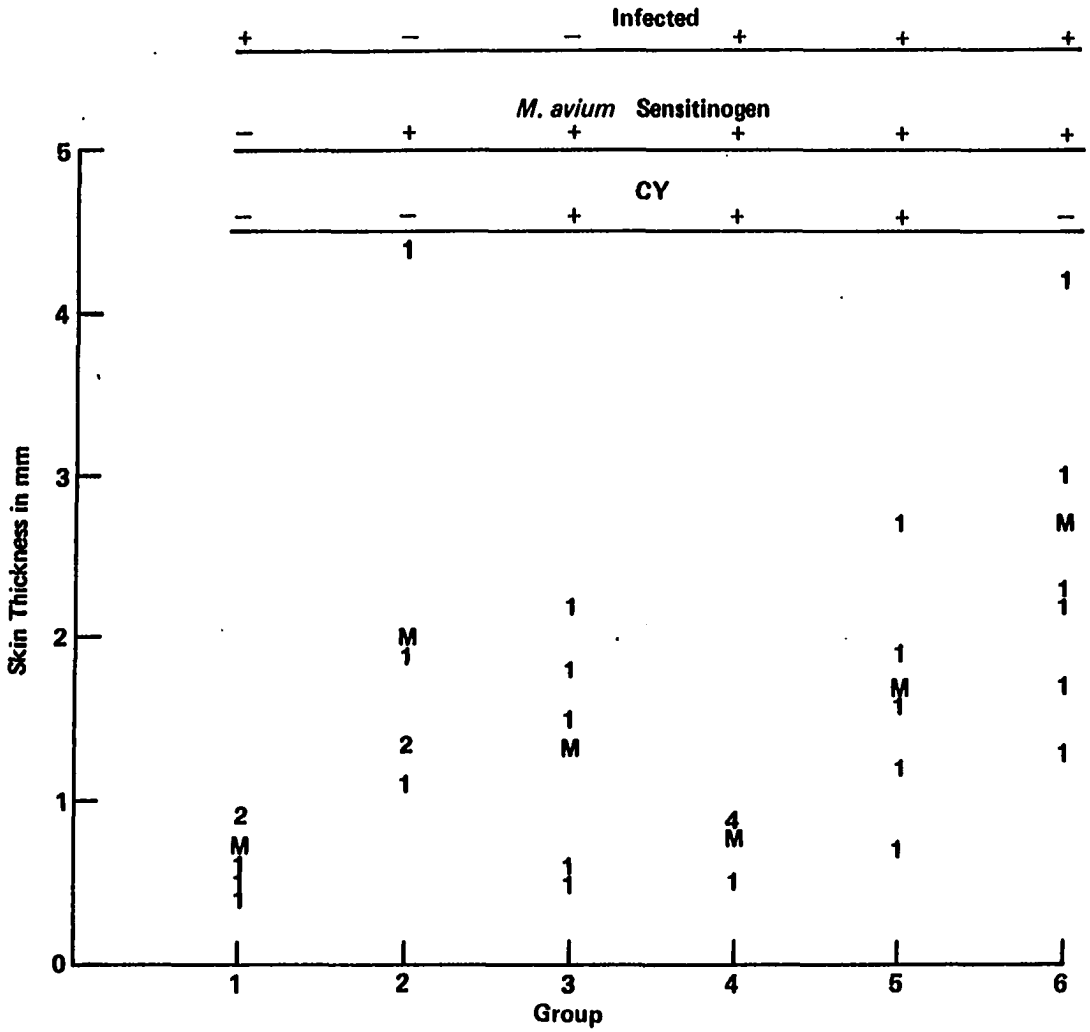

Figure 4. Maximal increase in skin thickness following intradermal injection of M. avium ppd. Numbers indicate the number of pigs with a given increased thickness. M = mean for each group of 5 pigs.

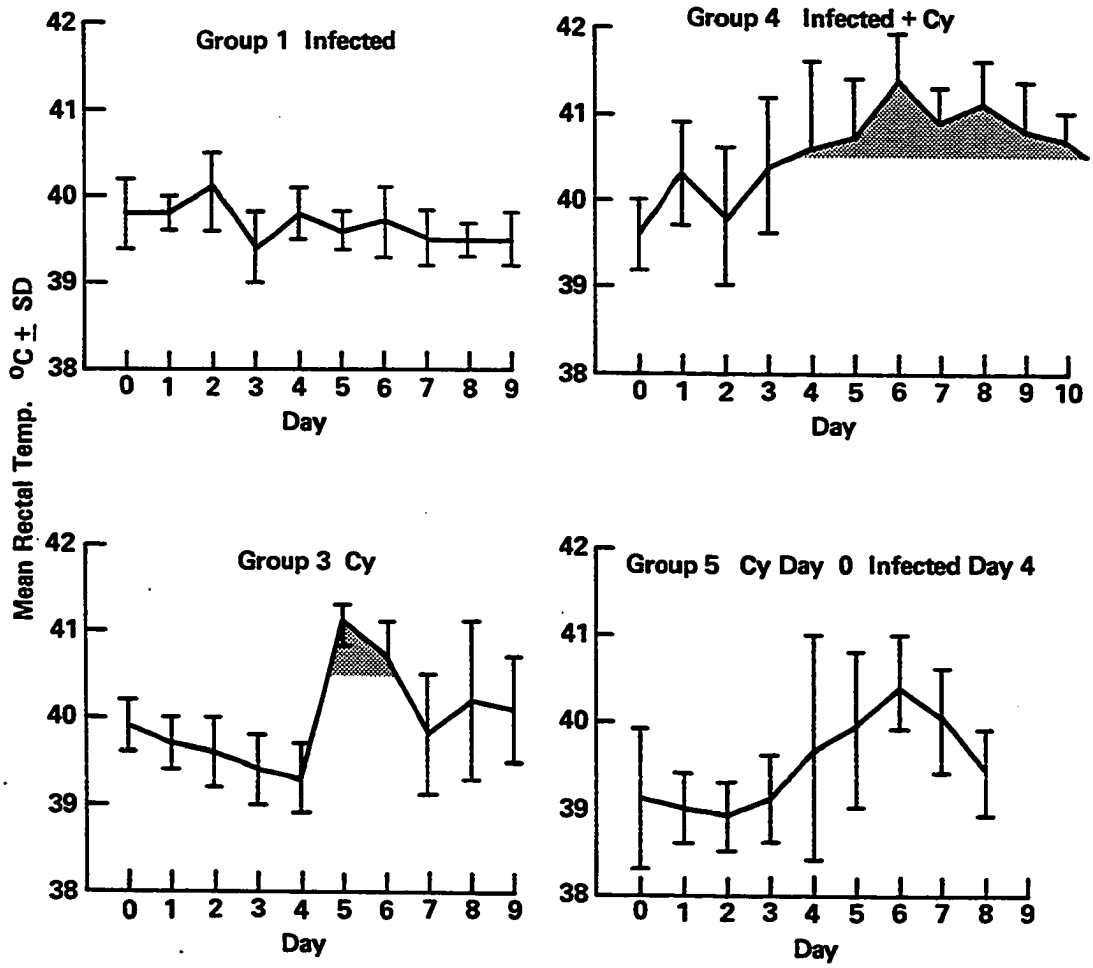

Figure 5. Mean daily rectal temperatures of pigs receiving cyclophosphamide and/or infected with Salmonella cholerae-suis strain 61. Shaded area is that above 40.5°C (104.9°F).

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EVALUATION OF MUTANTS OF *SALMONELLA CHOLERAE-SUIS* VAR *KUNZENDORF* FOR VIRULENCE AND IMMUNOGENICITY IN MICE

SUMMARY

Five strains of Salmonella cholerae-suis var kunzendorf were evaluated for immunogenicity and virulence in mice. Mutants of the strains were isolated and evaluated on a similar basis. Four of the five prototrophs (9, 33, 51 and 61) were avirulent for mice by the intraperitoneal route in a dose of 10^6-10^7 organisms. The fifth prototrophic strain (38) was highly virulent for mice at doses above 10^3 organisms. Vaccination with three of the avirulent prototrophs (33, 51 and 61) protected 50-60% of the mice against strain 38 challenge. Thymine-requiring mutants obtained through trimethoprim selection were also relatively avirulent except for strain $38T_1$. This auxotroph of strain 38 killed 5 of 10 mice. Protection provided by the thy^- mutants was equal to or greater than that afforded by the corresponding prototroph. An aro^-galE mutant of strain 38 (SL2808) was found to be completely avirulent for mice. Approximately 70% of mice were protected against virulent strain 38 challenge.

INTRODUCTION

Generation of a protective immune response against bacterial infections is a topic of great concern to the livestock industry. Bacterins and various fractions of bacterial agents have met with some success.[1,2] However, those bacterial agents which are capable of surviving and multiplying intracellularly have been best controlled through the use of live, attenuated "vaccines".[3-5] The increasing use and sophistication of techniques for manipulation of genetic material has enabled the specific modification of bacterial pathogens with the goal of producing an immunizing agent.[6-10] Unfortunately, such specific modification has met with limited success partly because most auxotrophic mutants remain virulent.[11,12] Another reason is the reluctance (of the livestock industry) to introduce non-virulent auxotrophic mutants capable of reversion to the fully virulent prototrophic form. Hoiseth and Stocker[7] recently reported the generation of a non-reverting auxotroph of Salmonella typhimurium. This auxotroph possessed a defect in the aromatic biosynthetic pathway which prevented its growth unless provided a source of p-amino-benzoic acid or 2,3-dihydroxybenzoate. These compounds are virtually absent from mammalian tissues.

Other mutations to auxotrophy have shown great promise as immunizing agents but the instability of these auxotrophs has generated fear as to their safety. Among those showing potential are the galactose epimerase mutants (galE)[6,13-15] and thymine or thymidine requiring mutants.[10] Introduction of a dual mutation each with a

reversion frequency of 10^{-9} to 10^{-10} into a potential live immunizing agent would practically eliminate any chance of reversion to full virulence. Toward this goal, initial work was performed to evaluate the immunogenic potential of 5 strains of S. cholerae-suis var kunzendorf which had been previously described.[16] Thymine-requiring mutants of each of the 5 strains were isolated through trimethoprim selection and the immunogenicity and virulence of the mutants compared with the parent strains. In addition, an aro$^-$galE mutant of a virulent strain of S. cholerae-suis was evaluated for virulence and immunogenicity in mice.

MATERIALS AND METHODS

Bacteria

Auxotrophic mutants of S. cholerae-suis strains 9, 33, 38, 51 and 61 were used in this work. Mouse-virulent strain 38 was used as a challenge strain. This strain produced essentially 100% mortality in mice at levels above 10^3 organisms given intraperitoneally. SL2808 an aro$^-$galE auxotroph of strain 38 was kindly supplied by Dr. Bruce A. D. Stocker. This auxotroph contained the aroA554::Tn10 transposon derived from S. typhimurium strain LT2 by transduction with phage $P_1C_mC_{t-s}$. Thymine requiring mutants $9T_1$, $33T_1$, $38T_1$, $51T_1$ and $61T_1$ were obtained from the prototrophic strains by trimethoprim selection. The procedure used was basically that of Stacey and Simson.[17] Briefly, the strains were grown 18 hr at 37°C in Vogel-Bonner E broth containing 0.5% glucose and 0.5% casamino acids (VB broth). One-tenth ml was transferred to 4.5 ml of VB broth containing 20 ug/ml thymidine and either 10 or 20 ug/ml trimethoprim. The tubes were wrapped in aluminum foil and incubated at 35°C for 4 days (until visibly turbid). Each culture was streaked on BHI agar containing 20 ug/ml thymidine (BHI$^+$) and incubated 24 hr at 35°C. Two or three colonies were subcultured to BHI$^+$. Thymine or thymidine requirement was checked by suspending cells from the BHI$^+$ in saline, spreading a lawn on VB agar (without thymidine) and spotting discs soaked in either thymine or thymidine onto the agar surface. All mutants were stored frozen at -80°C in sterile egg yolk. For use as an immunizing agent, the cultures were streaked onto 5% bovine blood agar, (BA) plated, and

incubated 24 hr at 35°C. Single colonies were picked to trypticase soy broth (TSB) and grown overnight at 35°C. Cultures were adjusted spectrophotometrically to OD 0.2 at 540 nm and diluted in TSB. Colony counts were enumerated by serial dilution and plating on 5% BA. Reversion frequencies varied from 10^{-8} to 10^{-10} for all thy$^-$ mutants.

Prototrophs

S. cholerae-suis strains 9, 33, 38, 51 and 61 were stored and grown as previously described.[16]

Mice

Sprague-Dawley[a] mice weighing 20-25 g were used throughout this work.

Experimental

In experiment 1, mouse virulence of strains 33, 51 and 61 was established. Groups of 10 mice were vaccinated intraperitoneally with 10^6 to 10^7 organisms and observed for mortality over a 3 week period. Mice were challenged intraperitoneally at 3 weeks post vaccination with strain 38 and observed for mortality for 3 weeks.

In experiment 2, mice were vaccinated intraperitoneally with thy$^-$ mutants $9T_1$ through $61T_1$ at a level of 10^4 to 10^7 viable bacteria per mouse. Strain $38T_1$ was evaluated at both 10^4 and 10^7 organisms/mouse.

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[a]Harlan Sprague-Dawley, Madison, WI.

Surviving mice were challenged at 3 weeks post-vaccination with 6.6 x 10^7 strain 38 and observed for mortality for 3 weeks. At the end of this time, mice were killed by cervical dislocation and the spleens cultured for salmonellae.

In experiment 3, two groups of mice were vaccinated with the aro$^-$ galE SL2808 and observed for mortality. At 2 weeks, these mice were challenged with 1.5 x 10^6 strain 38 intraperitoneally and observed for mortality.

RESULTS

Strain 38

Strains 33, 51 and 61 were found to be avirulent for mice even at a relatively high dose of 10^6-10^7 organisms (Table 1). No mortality was recorded for any of these strains during the three week period following vaccination. After challenge with strain 38, 10 of 21 (strain 33), 5 of 10 (strain 51), and 4 of 10 (strain 61) died. Earlier work with strain 9^{16} had indicated this to be an avirulent strain and it was therefore included in the group of 5 strains for which thy$^-$ mutants were obtained.

Mice vaccinated with strains $9T_1$ through $61T_1$ had little or no mortality except for the group receiving 10^7 strain $38T_1$ (Table 2). In this group, 5 of 10 mice died. The only other group with any mortality was that receiving strain $51T_1$. When challenged with strain 38 mortality appeared to be reduced from that observed in experiment 1. Only 2 of 12, 1 of 9 and 2 of 10 mice died that had been vaccinated with strains $33T_1$, $51T_1$ and $61T_1$ respectively. Mice surviving the 10^7 $38T_1$ vaccination were fully protected against the virulent prototroph. Positive spleen cultures were obtained for all groups except those vaccinated with $33T_1$ and 10^4 $38T_1$.

SL2808 proved to be avirulent for mice (Table 3). However, protection against the parent strain 38 was relatively poor. Three of 10 and 4 of 12 mice died in those groups receiving 10^5 and 10^6 SL2808 respectively.

DISCUSSION

In any live immunizing agent there exists a balance between virulence for the host and the degree of protection afforded against challenge. With some exceptions, organisms devoid of virulence tend to be less immunogenic; those which are highly immunogenic tend to be more virulent.[18,19] Rough strains of Salmonella although apparently safe have provided less than optimum levels of immunity.[20] It is thought that the extreme susceptibility of the rough organism to killing by complement and other serum factors accounts for the generally poor results. In addition, rough forms may quantitatively or qualitatively lack certain immunogens.

An ideal situation would be the use of a strain of organism possessing a full complement of the necessary antigen, capable of surviving and multiplying well in vivo, and which dies out after an optimum number of replications. UDP-galactose 4-epimeraseless (galE) mutants are thought to possess all of these qualities.[13] They are able to obtain enough galactose in vivo to supply the need for complete lipopolysaccharide formation. Without an exogenous source of galactose these mutants are able to form only a rough cell wall. In vivo galE mutants are capable of multiplying to high titer and in some cases have been known to be fully virulent depending on the degree and position of genetic defect. In addition, as a result of the defect in the epimerase, galactose cannot be metabolized and accumulates in the form of galactose-1-phosphate and UDP-galactose which causes lysis of the growing cells. Such galE mutants have been used for immunization

against typhoid[14] and have been widely evaluated experimentally in mice[19] and chickens.[8,10] A major drawback of this and other mutants is the capability of reversion to a fully virulent state. Formation of a double mutation effectively blocks such reversion. Attempts to use a double mutation within the galactose pathway have merely led to the formation of a rough but very stable mutant with immunogenicity equivalent to any other rough strain. It was believed that the combination of a gal E mutation with another mutation which is itself capable of reducing virulence while maintaining immunogenicity would be a potentially fruitful area of investigation. Towards this end, we initiated work to evaluate the effects of the thy$^-$ and aro$^-$ mutations on various strains of S. cholerae-suis. Our results indicate that the thy$^-$ mutant of S. cholerae-suis strain 38 was reduced in virulence. This is similar to work reported by others.[10] When injected into mice, mutants $33T_1$, $51T_1$ and $61T_1$ maintained and possibly increased the level of immunity provided when compared to the prototrophic strain (Table 2).

The aro$^-$galE mutant SL2808 proved to be avirulent (Table 3) in sharp contrast to the parent strain 38 which is almost universally fatal at doses above 10^3 organisms. However, the protection provided was only moderate to weak against challenge with the parent strain by the intraperitoneal route. It has been documented however, that the level of protection provided against an enteric pathogen increases when the live immunizing agent is administered orally instead of parenterally.[10] It is hoped that oral administration of SL2808 would

improve the protection provided. In addition, a reduction in challenge dose from 10^6 organisms would seem appropriate in light of the virulence of strain 38 for mice.

Table 1. Vaccination of mice with viable S. cholerae-suis prototrophs followed by challenge with S. cholerae-suis Strain 38

Strain	Vaccination dose	No. dead	Strain 38 challenge dose	No. dead
33	10^7	0/21	10^5	10/21
51	10^7	0/10	10^5	5/10
61	10^6	0/10	10^5	4/10

Mice were vaccinated and challenged via the intraperitoneal route. Challenge with Strain 38 followed by 3 weeks.

Table 2. Vaccination of mice with thy$^-$ mutants and challenge with S. cholerae-suis Strain 38

Mutant strain	Vaccination dose	No. dead post vaccination	No. dead post vaccination	No. positive spleen cultures
$9T_1$	10^5	0/10	3/10	3/7
$33T_1$	10^5	0/12	2/12	0/9
$38T_1$	10^4	0/10	2/10	0/8
$38T_1$	10^7	5/10	0/5	4/5
$51T_1$	10^7	1/10	1/10	6/9
$61T_1$	10^5	0/10	2/10	7/8

Mice were vaccinated and challenged by the intraperitoneal route. Challenge was with 6.6×10^7 Strain 38 three weeks post vaccination.

Table 3. Survival of mice vaccinated with SL2808 and challenged with S. cholerae-suis Strain 38

	SL2808 vaccine dose	No. dead of SL2808	Challenge dose strain 38	No. dead
Group 1	2.0×10^5	0/10	1.5×10^6	3/10
Group 2	2.0×10^6	0/12	1.5×10^6	4/12

Mice were vaccinated and challenged by the intraperitoneal route. Challenge followed vaccination by 14 days.

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